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Comunidades bacterianas estuarinas: diversidade filogenética e resistência a β-lactâmicos

Bacterial communities from an estuarine system: phylogenetic diversity and resistance to β-lactams

tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Prof. Doutor António Carlos Matias Correia, Professor Associado do Departamento de Biologia da Universidade de Aveiro e da Prof. Doutora Maria José Félix Saavedra, Professora Associada do Departamento de Ciências Veterinárias da Universidade de Trás-os-Montes e Alto Douro.

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resumo

As comunidades microbianas são extremamente complexas, estruturadas e compostas por microrganismos com características grandemente diversificadas. Estudos recentes permitiram esclarecer que a maioria dos microrganismos que constituem estas comunidades não são cultiváveis em condições laboratoriais. Por outro lado a possibilidade da definição de *taxa* com base em aspectos moleculares permitiu a construção de árvores filogenéticas que englobam e relacionam todos os microrganismos inclusivamente os não-cultiváveis. Estes conhecimentos constituíram a base para o desenvolvimento de novas técnicas de estudo de comunidades microbianas complexas, aplicáveis a diversos ambientes naturais.

Neste trabalho foram usadas metodologias independentes do cultivo na caracterização da diversidade filogenética das comunidades de bacterioplâncton do estuário Ria de Aveiro.

Estas comunidades apresentam, em termos de composição, consideráveis semelhanças com comunidades previamente caracterizadas, de outros sistemas costeiros e estuarinos, geograficamente distintos. Alterações profundas foram detectadas entre a zona marinha-salobra do estuário e a zona de água doce. Os grupos filogenéticos dominantes na secção marinha-salobra do estuário (*Bacteroidetes*, *α-Proteobacteria* e *γ-Proteobacteria*) são substituídos por *Bacteroidetes*, *β-Proteobacteria*, *δ-Proteobacteria* e *ε-Proteobacteria* na secção do estuário com salinidade próxima de zero. Foram também detectadas alterações sazonais. Verificou-se que as variações de salinidade e temperatura são responsáveis por uma parcela considerável das alterações detectadas na composição das comunidades bacterianas ao longo do gradiente estuarino.

Foram também aplicadas metodologias não dependentes do cultivo para aceder à diversidade de genes de resistência a antibióticos presentes neste estuário e que representam risco considerável em termos de saúde pública. Os métodos utilizados permitiram detectar uma elevada diversidade de sequências homólogas de genes que codificam β-lactamases de relevância clínica. A análise destas sequências sugere que este ambiente estuarino constitui um importante reservatório deste tipo de genes.

Métodos baseados no cultivo de microrganismos foram utilizados no sentido de obter dados complementares e permitiram confirmar a ocorrência de β-lactamases neste estuário e também a prevalência de integrões.

Os resultados obtidos durante este estudo evidenciam que os métodos baseados no cultivo não permitem aceder à totalidade do potencial genético de um ambiente natural e introduzem erros consideráveis neste tipo de análise. O estudo de comunidades bacterianas complexas deve, sempre que possível, combinar resultados obtidos através da utilização de vários métodos não dependentes do cultivo e métodos tradicionais baseados no cultivo dos microrganismos.

keywords

microbial communities, phylogenetic diversity, culture-independent methods, antibiotic resistance, β-lactamases, *Ria de Aveiro*

abstract

Prokaryotic communities are extremely complex and structured entities, composed by extremely abundant and diverse members. Recent advances such as the awareness of the existence of an unculturable prokaryotic majority, and growing progresses on taxonomy based on DNA sequences as well as the establishement of the sequence-based tree of life constituted the support for the development of novel culture-independent molecular techniques that offered new ways of studying microorganisms in diverse environments.
In this study culture-independent approaches were applied to characterise the phylogenetic diversity of bacterioplankton communities from *Ria de Aveiro*. The molecular phylogenetic analysis revealed a prokaryotic diversity comparable to other geographical distinct coastal and estuarine environments previously studied. Compositional shifts within this community occurred essentially between the brackish and freshwater sections. Seasonally driven changes in microbial community in this estuary also occur. The dominant bacterial groups changed from *Bacteroidetes*, *α-Proteobacteria* and *γ-Proteobacteria* in the marine-brackish section to *Bacteroidetes*, *β-Proteobacteria*, *δ-Proteobacteria* and *ε-Proteobacteria* in the freshwater section of the estuary. Results suggested that salinity and temperature fluctuations accounted for a significant amount of the phylogenetic variability detected along the estuarine gradient.
Culture-independent methodologies were also applied to study the diversity of genetic molecular determinants of antibiotic resistance within this estuary, which potentially constitute a risk to human and ecological health. Sequences representing clinical relevant families of β-lactamases were detected in *Ria de Aveiro*. Most of the retrieved sequences were identical or very similar to β–lactamase gene sequences previously characterised from clinical isolates. Phylogenetic analysis suggests that this aquatic ecosystem is a reservoir of molecular diverse putative *bla* sequences. Culture-dependent approaches were applied to obtain complementary data on this subject. Considerable levels of prevalence and diversity of β-lactamase genes and integrons were confirmed to occur in *Ria de Aveiro*.
This study reinforces the hypothesis that cultivation-dependent approaches are insufficiently adequate to study the phylogenetic and functional molecular diversities of bacterial communities from natural environments. Taken together obtained results suggested that, whenever possible, complex microbial communities should be studied by using a combination of different culture-independent methodologies and, when necessary, cultivation-based methods must be applied to the same samples to obtain complementary data.

List of original publications

This thesis includes results published in the articles listed bellow. Additionally, some unpublished results are presented.

Henriques I, Almeida A, Cunha A & Correia A (2004) Molecular sequence analysis of prokariotic diversity in the middle and outer sections of the Portuguese estuary *Ria de Aveiro*. *FEMS Microbiology Ecology* 49(2): 269-279. – Results presented in **Chapter 3**.

Henriques I, Alves A, Tacão M, Almeida A, Cunha A & Correia A (2006) Genetic diversity and dynamics of free-living bacterial community along an estuarine gradient (*Ria de Aveiro*, Portugal). *Estuarine Coastal and Shelf Science* 68, 139-148. – Results presented in **Chapter 4**.

Henriques I, Moura A, Alves A, Saavedra MJ & Correia A (2006) Analysing diversity among beta-lactamase encoding genes in aquatic environments. *FEMS Microbiology Ecology* 56, 418-429. - Results presented in **Chapter 5**.

Henriques I, Fonseca F, Alves A, Saavedra MJ & Correia A (2006) Occurrence and diversity of integrons and beta-lactamase genes among ampicillin-resistant isolates from estuarine waters. *Research in Microbiology* (*submitted*). – Results presented in **Chapter 7**.

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Isolation Kit (lanes 1, 2 and 3) and Genomic DNA Purification Kit (lanes 4, 5 and 6). A positive control was included (lane 7). Lane 8: DNA size marker GeneRuler™ 100 bp DNA ladder plus (MBI Fermentas, Vilnius, Lithuania).

Figure 5.2 DGGE analysis of β-lactamase gene fragments amplified from clones (lanes 1-24) picked from TEM (A), IMP (B) and OXA-B (C) genomic libraries. Lanes C, fragment amplified from positive controls of the corresponding β-lactamases. In Figure 5.2A the band positions T1, T2 and T3 are indicated. For Figure 5.2B the correspondences between band positions and clones are: band position IM1 - clones 1, 3, 10; IM2 - 4, 6, 8; IM3 – 5; IM4 – 7, 20; IM5 – 9; IM6 - 2, 12, 14, 15, 16, 19; IM7 – 13, 22; IM8 - 21, 23, 24 and IM9 - 11, 17, 18; for Figure 5.2C the correspondences are band position O1- clones 2, 4, 5, 6, 7, 8, 10, 11, 12, 15, 16, 17, 19, 20, 23, 24; O2 – 9; O3 - 1, 3, 13, 18, 21; O4 - 14 and O5 - 22. Pag. 92

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6. Genotyping and identification of ampicillin-resistant isolates from estuarine waters

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7. Occurrence and diversity of integrons and β–lactamase genes among ampicillin-resistant isolates from *Ria de Aveiro*

1. *Introduction*

1.1 Environmental microbial communities: remarkable complexity

The study here presented deals with phylogenetic and functional diversity of prokaryotes, meaning unicellular microorganisms characterised by the lack of a distinct membrane-bound nucleous. In this revision emphasis will be put in the features of prokaryotes and prokaryotic communities, however occasionally the general designations "microbes" and "microbial communities" will be applied.

Despite many outstanding technological advances, our comprehension of the prokaryotic world remains very incomplete and controversial. Recently, several studies regarding the quantification of the abundance and diversity of *Bacteria* and *Archaea* in a vast array of natural environments were published. Whitman and co-workers (1998) estimated the total number of prokaryotes in earth to be around 10^{30} cells. Specifically, average cellular densities of 10^{6} cells per millilitre of sea water and of 10^{10} cells per gram of soil have been reported. These authors concluded that prokaryotic biomass accounts for more than half of the total biomass on our planet.

Regarding phylogenetic diversity, Curtis and co-workers (2002) developed a strategy that allowed estimating that a millilitre of sea water contains around 160 microbial "species"[1] and a gram of soil contains approximately 6300 "species". Similar values had been reported previously by Torsvik and co-workers (1990). The accuracy of this numbers is supported by molecular-based studies developed on water samples from The Sargasso Sea (Venter *et al*., 2004) and on soil samples (Lunn *et al*., 2004).

In addition to the huge values reported for microbial abundance and diversity, it is also known that the extremely diverse metabolic characteristics of microbes allow them to inhabit and function in remarkably different habitats. Prokaryotes can be found in environments which offer normal conditions for life development such as soils, lakes, rivers, estuaries, oceans and other living organisms, but they survive also in environments usually referred as "extreme environments". In fact, prokaryotes have been isolated or detected in hydrothermal vents (at temperatures as high as 113 ºC), in the liquid phase of sea ice (at temperatures as low as -20 ºC) in acidic mine waters and soda lakes (with pHs

[1] Curtis and co-workers stated: "The work does not presuppose a particular definition of a species, merely the existence of credible criteria for distinguishing between different organisms. For the purpose of this paper, this means a meaningful difference in the sequence of the 16S RNA gene."

as low as 0.7 and as high as 11, respectively), in salt flats and marine hypersaline basins (with salinities of about 30 %) and kilometres below the Earth's surface (Blochl *et al*., 1997; Deming, 2002; Horner-Devine *et al*., 2004; Irwin & Baird, 2004; White *et al.*, 1998).

Because of prokaryotes immense abundance, diversity and ubiquity, they play crucial functions in the planet's biogeochemical processes such as primary production and consumption of organic matter, cycling of nutrient elements, regulation of the atmosphere composition, nitrogen fixation, and photosynthesis (Doney *et al*., 2004). In fact, the key functional roles prokaryotes play, may imply that in their absence other forms of life would also cease to exist.

Even though our knowledge of prokaryotic diversity has increased greatly in the recent decades, novel scientific reports regularly highlight the limitations of that understanding. The acknowledgement that a microbe does not exist by himself added obstacles to an already difficult task. Microbial communities are composed by structured clusters of microbial species, playing different and complementary roles (Rodriguez-Válera, 2002; Torsvik & Ovreas, 2002). Characteristics of a microorganism determined in the laboratory most of the times do not reflect its real properties in the environment.

Culture-independent analysis (further discussed in detail in section 1.2.4 of this thesis) highlighted the enormous complexity of natural bacterial and archaeal communities in terms of phylogeny and function (Amann *et al*., 1995; Giovannoni *et al*., 1990; Handelsman, 2004; Head *et al*., 1998; Rappé & Giovannoni, 2003; Ward *et al*., 1990). Furthermore, several studies confirmed the assumption that microbial communities composition and structure is highly influenced by spatial and temporal environmental variables such as temperature (**Chapter 4**[2]; Panswad *et al*., 2003), precipitation (Bernhard *et al*., 2005), salinity (**Chapter 3**; **Chapter 4;** Bernhard *et al*., 2005), nutrient status (Mills *et al*., 2003), contamination with pollutants (Li *et al*., 2006) and several others.

In summary, prokaryotic communities are extremely complex and structured entities, composed by extremely abundant and diverse members, which play key roles in the maintenance of life conditions on our planet. This enormous complexity has

[2] Whenever appropriate research results obtained during this study are cited in the text, reporting to the chapter where they are presented.

prevented, for decades, significant progresses in Environmental Microbiology[3] and Microbial Ecology[4].

In fact, the extensive cataloguing of microbial species and microbial functions in a microbial community has been considered virtually impossible by several authors (Brock, 1987; Wilson, 1994). Wilson (1994) actually stated that microbial diversity is "beyond practical calculation". However, in the last decades, outstanding methodological improvements have been achieved and promising results were obtained.

Microbiologists are now aware that it is essential to understand how transformations in microbial communities affect ecosystem functioning and human life and how human activities affect microbial communities. To achieve this state of knowledge, a great effort must be made to unravel the functional and phylogenetic diversity of prokaryotes in specific natural environments, as well as the environmental and anthropogenic factors determining these diversities.

1.2 Studying microbial communities: assessing the inaccessible

As stated before, when studying diversity within a microbial community several different aspects should be assessed. Among these, microbial phylogenetic diversity (catalogue of the prokaryotic species that compose a given community) and microbial functional diversity (physiologic and metabolic capabilities of the community members) obviously are key points to properly describe a microbial community (Schloss & Handelsman, 2004).

For decades microbiologists have developed their studies based on traditional tools such as microscopy, staining methods and pure-cultures (Brehm-Stecher & Johnson, 2004). However, bacterial shapes are usually ordinary rods or spheres, being almost impossible to establish differences between microorganisms based on morphology. Other traits such as biochemical properties and metabolic activities most of the times are very

[3] Environmental microbiology is the study of microorganisms which exist in natural or artificial environments (Hurst, 2002).

[4] Microbial ecology studies microbial communities and how these communities interact with their environment (abiotic and biotic) (Brock, 1987).

unstable, changing according to environment-adaptation, and so they do not provide a robust frame for classification of the microorganisms. Besides, this last approach requires the isolation and cultivation of individual cells. As discussed in the next section, microorganisms that grow under laboratory conditions may not be representative, or even not the major components, of the prokaryotic community of which they are natural members (Rodriguez-Valera, 2002). For the above mentioned reasons, molecular-based methods are, nowadays, considered essential to characterise microorganisms and microbial communities.

1.2.1 The unculturable majority

In the early decades of the 20th century, microbiologists were not aware of the unculturability of certain microorganisms and even believed that almost all prokaryotic diversity had already been studied. In the 1st edition of the *Bergey's Manual of Systematic Bacteriology* published in 1923, it was stated that a microorganism could not be classified without being cultured and Waksman and Starkey, in 1931, stated that "a large body of information has accumulated that enables us to construct a clear picture of the microscopic population of the soil".

The first reports highlighting the impossibility to cultivate the majority of the environmental microorganisms resulted from the so-called "great plate count anomaly", meaning the evident discrepancy between numbers of microorganisms estimated by plating and by microscopy (Jannasch & Jones, 1959; Kogure *et al*., 1979; Staley & Konopka, 1985). A number of studies estimated that in aquatic environments calculations of colony forming units (CFUs) and microscope counts can differ by four to six orders of magnitude (Grimes *et al*., 1986) and in soil only 0.1 to 1 % of bacteria were found to be readily cultivable under standard laboratory conditions (Table 1.1; Torsvik & Ovreas, 2002). Additionally, other studies highlighted the viability and importance of the unculturable microorganisms in the natural ecosystems (Colwell *et al*., 1996; Marshall *et al*., 1985).

The detailed reasons why most existing microorganisms cannot be cultivated are not entirely understood, but in general this results from the impossibility to mimic the natural conditions, using instead laboratory highly artificial and restrictive growth conditions (Barer & Harwood, 1999). Clearly, the employment of culture-based

methodologies introduces biases not only due to the unculturability of most microorganisms but also to the unpredictable different levels of culturability of different phylogenetic groups. Even closely related microorganisms may show very different capacities to adapt to laboratory conditions. For example, despite the fact that *Mycobacterium leprae* is undoubtedly closely related to *M. tuberculosis*, the first one seems to be recalcitrant to cultivation (Kramme, *et al*., 2004).

Table 1.1 Culturability determined as a percentage of culturable bacteria (CFU) in comparison with total cell microscopy-based counts (adapted from Amman *et al*., 1995).

Habitat	Culturability (%)
Seawater	0.001-0.1
Freshwater	0.25
Mesotrophic Lake	0.1-1
Unpolluted estuarine waters	0.1-3
Activated sludge	1-15
Sediments	0.25
Soil	0.3

As it would be expected, most of the knowledge about microorganisms now available comes from the study of culturable strains. For example, approximately 65 % of the total microbiological studies published between 1991 and 1997 concerned the study of only eight, easily culturable, bacterial genera (members of only three bacterial phyla, namely *Proteobacteria*, *Firmicutes* and *Actinobacteria*): *Escherichia* (18 %), *Helicobacter* (8 %), *Pseudomonas* (7 %), *Bacillus* (7 %), *Streptococcus* (6 %), *Mycobacterium* (6 %), *Staphylococcus* (6 %) and *Salmonella* (5 %) (Galvez *et al*., 1998). Obviously, this small number of different genera cannot be representative of the prokaryotic diversity.

Floyd and co-workers (2005) analysed the *American Type Culture Collection* (ATCC) to determine how described bacterial species reflects the general tendencies in microbial diversity in different habitats. The authors concluded that in most of the analysed environments there is a significant discrepancy between the phylogenetic types that are

predominant in the culture collection and the phylogenetic types that were retrieved in studies applying culture-independent methodologies. Specifically the phyla *Acidobacteria*, *Verrucomicrobia*, and *Planctomycetes* were found to be particularly underestimated in this culture collection (Floyd *et al*., 2005).

In fact, it is generally recognised that prokaryotic diversity remains almost unexplored. The current description of approximately 6600 validly named species of bacteria (Floyd *et al*., 2005) constitutes an almost insignificant number when compared to the estimated diversity of, for example, 6300 species per gram of soil (Curtis *et al*., 2002).

1.2.2 The species concept

Assessing microbial phylogenetic diversity is obviously interesting by itself, but additionally allows inferences about the functional diversity of microbial communities. The first problem when studying microbial phylogenetic diversity derives from the fact that the species concept in bacteria has always been, and still is, controversial (Doolittle, 2002; Ródriguez-Valera, 2002; Shimwell & Carr, 1960). The biological concept of species was first applied to plants and animals, organisms with sexual reproduction; however, bacteria normally reproduce by binary partition (except for partial genetic transfers). Difficulties result from the basic clonal structure of bacterial populations and from the confirmed potential of genetic exchange between distantly related phylogenetic groups (Ochman *et al*., 2005).

Rodriguez-Valera (2002) stated that "Rather than well-defined evolving units, bacterial species might be sort of skeletons or frames of genes in which sizeable clusters of genes easily exchangeable such as transposons, phages or integrons, are assembled to produce a phenotype adapted to a specific ecological niche." Nowadays, to define species, molecular properties such as DNA-DNA hybridization and 16S rRNA gene sequences are usually selected (Coenye *et al*., 2005; Ochman *et al*., 2005; Rodriguez-Valera, 2002).

DNA-DNA hybridization has been considered the gold standard for delineation of prokaryotic species and remains a frequently applied technique; 70% constitutes the

traditional DNA–DNA re-association level for species definition. However this technique is characterized by its laborious nature and lack of reproducibility (Ward & Fraser, 2005).

On the other hand 16S rDNA sequence analysis is a much simpler and now frequently used criterion for phylogenetic affiliation, despite the recognised different rates of evolution displayed by different phylogenetic clusters (some genera show less divergence than distantly related species of a single genus) (van de Peer *et al*., 1996). Stackebrandt and Goebel (1994) recommended a cut-off of 3% divergence in the 16S rRNA genes for separating species. This criterion has greatly facilitated the study of diversity within microbial communities.

In recent years, sequence analysis of other housekeeping genes, individually or in combination, has been used for the same proposes (Cohan, 2002). Also, the advent of completely sequenced genomes allowed the construction of molecular-based phylogenies more reliable than single-gene-based trees (Coenye *et al*., 2005).

In conclusion, phylogenetic diversity can now be inferred even when the phenotypic diversity is unknown. In the case of phylogenetic analysis of uncultivable bacteria, molecular techniques are the only applicable and valid approaches (Cohan, 2002).

Recently, new concepts were proposed to define a bacterial species, namely Cohan (2002) suggested that a bacterial species should not be defined simply as a cluster of phenotypically and genetically similar organisms but may alternatively be defined as groups having a set of dynamic properties (genetic, ecological, evolutionary, or phylogenetic). This entity was designated "Ecotype" and the author confirmed that "Ecotypes" were identifiable as monophyletic groups in a phylogeny based on DNA sequence data.

1.2.3 The universal tree of life

Carl Woese and colleagues developed an extensive work on comparative analysis of small-subunit ribosomal RNAs (16S and 18S rRNAs), which allow them to determine evolutionary relationships between organisms (Woese *et al*., 1990; Woese *et al*., 1985; Woese, 1987). The authors suggested that diversity can be ascertained as sequence divergence on a phylogenetic tree. Woese divided living organisms into three primary

domains (*Eucarya*[5], *Bacteria* and *Archaea*) and within the *Bacteria* domain he defined 11 major phyla or divisions (all based on 16S rRNA gene sequences obtained from cultivated organisms).

Woese work changed dramatically the study of microorganism and, as will be discussed later in this thesis, the study of microbial communities. The phylogenetic tree revealed relationships between genera phenotypically unrelated such as *Bacteroides* and *Flavobacterium*. The *Bergey's Manual of Systematic Bacteriology* abandoned the traditional phenotypic characterisation and adopted 16S rRNA criterions to classify prokaryotes (Ludwig & Klenk, 2001). Sequence surveys allowed the discovery of new bacterial taxa, including new divisions (Figure 1.1). Finally, based on Woese sequence-systematics it became possible to characterise the phylogenetic diversity among uncultured bacteria.

1.2.4 Molecular based cultivation-independent methods

The advances described in the above sections, namely the awareness of the existence of an unculturable prokaryotic majority, the development of a species-concept based on molecular data and the construction of the sequence-based tree of life, constituted the support for the development of novel molecular techniques that offered new ways of studying microorganisms in diverse environments.

Norman Pace and co-workers (1985) first proposed cultivation-independent approaches to study natural microbial populations[6]. Their strategy consisted in the analysis of 5S or 16S rRNA gene sequences directly in nucleic acids extracted from environmental samples, without culturing. The development of the PCR technology and the design of primers that can be used to amplify almost the entire genes facilitated the experimental performance of such analysis. Nowadays, PCR amplification of 16S rDNA

[5] "Additionally, "eukaryotes" will continue to be an acceptable common synonym for the *Eucarya*. However, we recommend abandonment of the term "archaebacteria," since it incorrectly suggests a specific relationship between the *Archaea* and the *Bacteria*." (Woese *et al*., 1990).

[6] "The simple morphology of most microbes provides few clues for their identification; physiological traits are often ambiguous. The microbial ecologist is particularly impeded by these constraints, since so many organisms resist cultivation, which is an essential prelude to characterisation in the laboratory" (Pace *et al*., 1985).

sequences from the environmental DNA, cloning of the resultant amplicons and comparison of the obtained sequences with sequences previously reported allows their assignment to a phylogenetic group within a phylogenetic tree.

This approach is now widely used to determine the phylogenetic diversity within natural microbial communities and dramatically improved the current knowledge on this topic. The first studies conducted using cultivation-independent approaches reported that the majority of the 16S rRNA gene sequences recovered from natural communities did not belong to already described species (Giovannoni *et al*., 1990; Ward *et al*., 1990). Until 2002, more than 29500 16S rRNA gene sequences (from the *Archaea* and *Bacteria* domains) obtained using cultivation-independent approaches have been published in GenBank, while only approximately 14000 16S rRNA gene sequences from cultivated microorganisms have been deposited until the same date (Rappé & Giovannoni, 2003).

In fact, the use of the approaches described firstly by Pace and co-workers has resulted in an increase of the number of recognised bacterial phyla. Hugenholtz and co-workers (1998) reported an increase from 11 phyla[7] in 1987 (Woese, 1987) to 36 in 1998, 13 of which did not contain cultured members. Recently, it was reported that the sequence census has resulted in a tree containing 53 phyla, 25 of which do not include culturable members (Figure 1.1; Handelsman, 2004). Furthermore, it is reasonable to predict that the probable increase in the number of 16S rDNA sequences reported each year will imply the redefinition of the currently defined phyla.

Some of the new described lineages were found to be dominant in a given environment, despite the fact that their members had been underestimated using culture-dependent methodologies. For example, the SAR11 clade of the *alpha-Proteobacteria*, which includes essentially unculturable members and only a few recently cultivated members (Rappé *et al*., 2002), is known to represent with the *Acidobacterium* phylum more than 25 % of the 16S rRNA sequences retrieved from several environments (Giovanonni *et al*., 1990; Ludwig *et al*., 1997). Also, phyla *Verrucomicrobia* and *Chloroflexi* are currently known to be ubiquitous but include only a limited number of cultivated microorganisms as members (Rappé & Giovannoni, 2003).

[7] The 11 phyla proposed by Woese were: green non-sulfur bacteria; deinococci and relatives; spirochetes; green sulfur bacteria; bacteroides-flavobacteria; planctomyces and relatives; chlamydiae; Gram-positive bacteria; cyanobacteria; purple bacteria and other phyla (Thermotogae) (Woese, 1987).

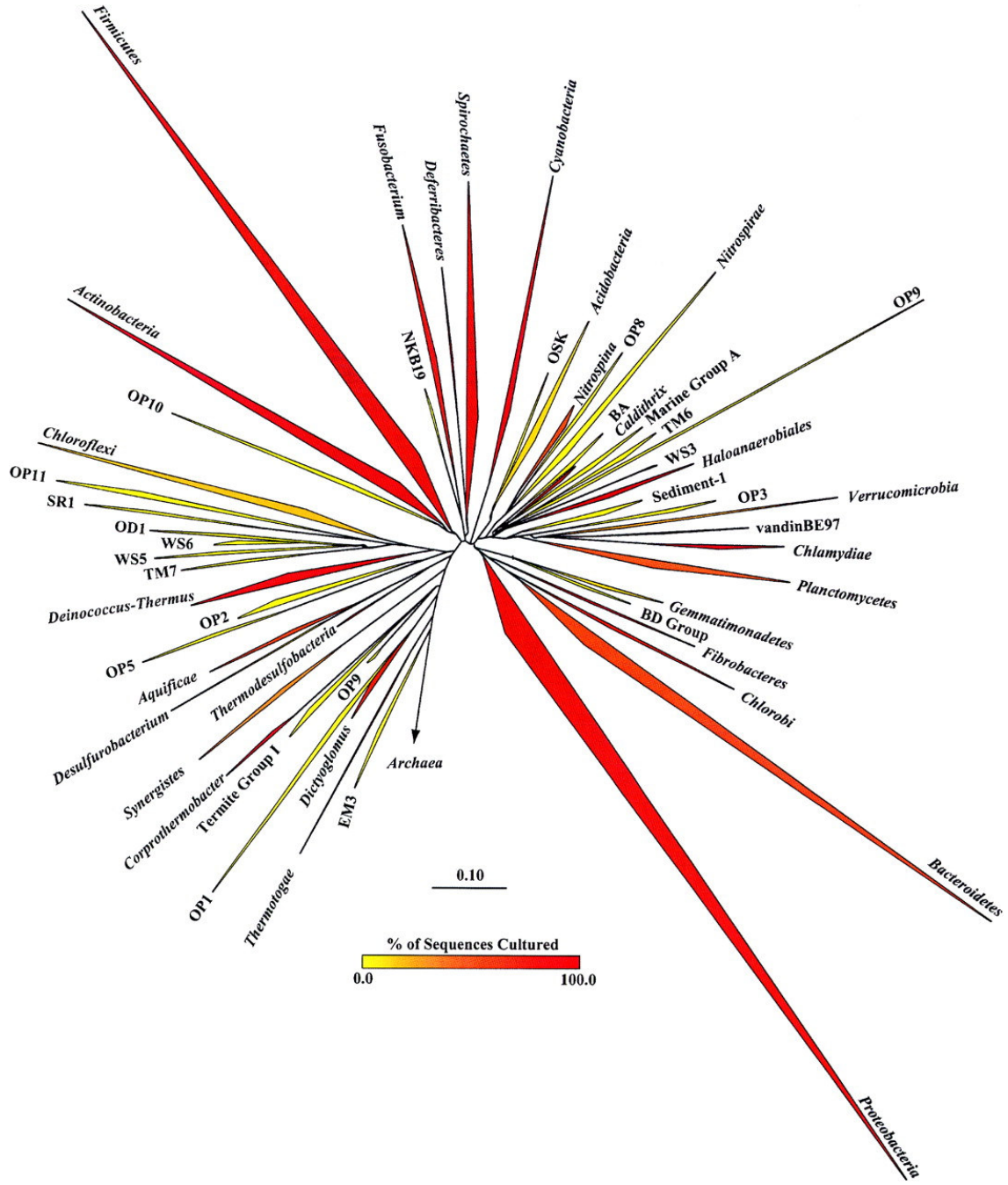

Figure 1.1 Phylogenetic tree as drawn by Handelsman (2004). The tree presents established phyla (italicised Latin names) and candidate phyla (which do not contain any cultured member) included in the domain Bacteria. The tree was built using 16964 16S rDNA sequences longer than 1000 bp. The vertex angle of each wedge indicates the relative abundance of sequences in each phylum; the length of each side of the wedge indicates the range of branching depth found in that phylum; the redness of each wedge corresponds to the proportion of sequences from cultured representatives in that phylum.

1.2.4.1 The 16S ribosomal RNA: a key molecule in Microbial Ecology

As stated in the above sections, the first described and most currently used molecular methodologies in Microbial Ecology rely on the employment of the ribosomal RNA genes as phylogenetic markers. There are several advantages why these molecules became central in this area, namely:

- the ribosomal RNAs are part of the protein-synthesising machinery, obviously essential for all organisms; thus, these molecules occur in all organisms with high levels of structural and functional preservation.

- their primary structure is divided into several different segments, which vary from extremely conserved (even between different phylogenetic domains) to extremely variable (van de Peer *et al*., 1996). Conserved segments can be used to study old relationships, whereas variable regions are useful to clarify evolutionary relationships between closely related organisms.

- the highly conserved segments allow rRNA sequences to be aligned and target sequences for the design of PCR primers or hybridisation probes to be detected (Giovanonni *et al*., 1988). Oligonucleotides can be designed based on the more conserved regions of the molecule and used as universal primers or probes, or on highly variable sequence regions and used as species-specific hybridisation probes or PCR primers;

- rRNA sequences are long enough to allow statistically significant phylogenetic analysis.

- rRNA genes are not (or are scarcely) objects of horizontal gene transfer between different organisms allowing evolutionary inferences from their analysis.

For all these reasons rRNAs and rRNAs encoding genes are usually the molecules of choice for establishing phylogenetic relationships. Especially the 16S rRNA sequence has been widely used due to the fact that its size (approximately 1500 nucleotides) provides sufficient information to design phylogenetic inferences and also because there is a vast number of currently available complete 16S rRNA sequences in the databases[8] making comparisons easier (Cole *et al*., 2005).

[8] For example, the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/) provides free ribosome related data and services to the scientific community, including online data analysis and aligned and annotated 16S rRNA sequences (Cole *et al*., 2005). The last release contains 210059 aligned and annotated bacterial small-subunit rRNA sequences, and is updated frequently.

1.2.4.2 Methodological details and current applications

The first applied culture-independent molecular approaches consisted in the construction and analysis of rDNA libraries from bulk DNA, extracted from environmental samples. More commonly 16S rRNA genes were amplified using a PCR-based approach. The amplified DNA fragments were then cloned into a vector and transformed into an *Escherichia coli* host. Taking advantage of the conserved regions within the target rRNA gene, "universal"[9] primers comprising complementary sequences to rRNA genes from all members of each domain were designed (**Chapter 3**; Giovanonni *et al*., 1990; Lane *et al*., 1985; Lane, 1991). Alternatively, specific phylogenetic groups of interest can also be surveyed using group-specific primers (Gyamfi *et al*., 2002; Holmes *et al*., 2000). In some cases the use of rRNA libraries (obtained through the use of reverse transcriptase) was considered useful to assess the most metabolically active members of the community (Nogales *et al*., 1999; Nogales *et al*., 2001).

Over the past decades, phylogenetic surveys based on the construction of 16S rDNA or 16S rRNA libraries have been conducted in a wide variety of artificial and natural environments. Several studies were reported on the characterisation of marine water microbial communities from, for example, the Sargasso Sea (Giovanonni *et al*., 1990; Venter *et al*., 2004), the Atlantic Ocean (Davey *et al*., 2001; Gallagher *et al*., 2004), the Pacific Ocean (Rappé *et al*., 2000) and the Artic Ocean (Bano & Hollibaugh, 2002), and also on the characterisation of microbial communities from marine sediments (Tal *et al*., 2005; Wang *et al*., 2004). A great amount of studies have also been performed on freshwater systems such as lakes and rivers (Cottrell *et al*., 2005; van der Gucht *et al*., 2005), on estuarine systems (**Chapter 3**; Kisand & Wikner, 2003; Kirchman *et al*., 2003), on soil (Nogales *et al*., 2001; Torsvik & Ovreas, 2002), on food samples (Handschur *et al*., 2005; Loy *et al*., 2005), and also on microbial communities associated to humans or animals (Daly *et al*., 2001; Wang *et al*., 2005).

[9] An increasing number of reports highlight the fact that 16S rDNA sequences of some newly recognised groups are very diverse and include mismatches to the commonly used "universal primers" (see for example **Chapter 3** and **Chaper 4**).

The described 16S rDNA approach can be brought full-cycle by applying specific 16S rRNA-targeted nucleic-acid probes specific to visualise and enumerate particular groups (identified and selected through the analysis of the 16S rDNA library) in the environmental sample, using techniques such as whole-cell fluorescence in situ hybridisation (FISH) (Amman *et al*., 1995; Juretschko *et al*., 2002). Another major advantage of the construction of 16S rDNA libraries is that almost the entire gene can be retrieved maximising the phylogenetic information obtained. However, to obtain representative results a high number of clones must be processed from the same sample (Kemp & Aller, 2004). Even though this screening can be facilitated by complementary approaches such as restriction fragment length polymorphism (RFLP) analysis (see for example **Chapter 3**), this process remains lengthy, costly, and time-consuming.

Therefore, many other culture-independent techniques have been developed to assess microbial community diversity. Fingerprinting methods, which allow monitoring the spatial and temporal dynamics of a microbial community, provide a suitable and fast alternative. Several culture-independent fingerprinting methods have been described, namely automated ribosomal RNA intergenic spacer analysis (ARISA) (Fisher & Triplett, 1999), terminal-restriction fragment length polymorphism (T-RFLP) (Avaniss-Aghajani *et al*., 1994), single-stranded conformational polymorphism separation of mixed rRNAS (r-RNA-SSCP) (Macgregor & Amann, 2006), temperature gradient gel electrophoresis (TGGE) (Muyzer & Smalla, 1998) and denaturing gel gradient electrophoresis (DGGE) (Muyzer *et al*., 1993).

For example, the extensively used DGGE technique separates different gene fragments based on their melting temperature, which is a function of their G+C content. The basic methodology comprises extraction of environmental DNA, amplification using PCR with primers targeting part of the gene and separation of the amplicons on a polyacrylamide gel with a gradient of increasing concentration of denaturants (formamide and urea). Usually a 35-40 bp clamp is attached to the 5′ end of the forward primer to prevent complete denaturation of the DNA fragment (Muyzer *et al*., 1993). DGGE has the advantages of being reliable, reproducible, fast and rather inexpensive, allowing the analysis of multiple samples simultaneously. This method is more helpful when used in combination with band sequencing, allowing dominant taxonomic groups to be qualitatively tracked in environmental samples.

Due to the above mentioned advantages, DGGE has been extensively applied in studies concerning the dynamics of microbial communities, which imply usually the analysis of a large number of samples. For example studies have been reported concerning the spatial and/or temporal dynamics of microbial communities from estuarine waters (**Chapter 4**; Crump *et al*., 2004), rivers (Feris *et al*., 2003), soils (Li *et al*., 2006), food fermentation processes (Fontana *et al*., 2005), bioreactors (Carvalho *et al*., 2006; Connaughton *et al*., 2006) and sewage treatment plants (Rowan *et al*., 2003).

DGGE and the other mentioned fingerprinting methods are inadequate for detection and quantification of rare species in an environmental sample. Recently a promising microarray-based technology was described by Palmer and coworkers (2006), which allowed the detection and quantification of species that are present in complex mixtures in abundances lower than 0.1 % in complex mixtures. Further investigations are needed to confirm the usefulness of this novel method for routine characterisation and monitorisation of microbial communities.

1.2.5 Molecular-based assessment of functional diversity

As stated in the above sections, molecular and genomic-based techniques are fundamentally changing our view of the prokaryotic world. Those techniques have proven useful for unravelling the phylogenetic diversity and organisation structures of microorganisms within microbial communities. However, fundamental questions about both physiologic and metabolic diversities still remain. Molecular techniques can also potentially contribute to solve such questions and are absolutely essential to assess functional diversity within unculturable microorganisms.

Studies of phylogenetic diversity based on 16S-rRNA/16S-rDNA approaches can provide insights into the functional diversity of the correspondent communities. This has been proved for specific functional groups such as ammonia-oxidising bacteria (Xia *et al*., 2005), sulphate-reducing bacteria (Kelly *et al*., 2005; Stubner, 2004) and nitrite-oxidising bacteria (Kelly *et al*., 2005), in which the phylogenetic composition determines the potential to perform specific physiological roles. However, most of the times, physiological abilities are spread across the phylogenetic tree while a single group can perform many concomitant functions. Additionally, many functional genes can be

transferred horizontally. In these cases, conclusions regarding the possible phenotypes, ecological behaviours and metabolic processes of the microbes cannot be drawn from 16S-rRNA/16S-rDNA analysis.

Several other culture-independent molecular methodologies have been applied to assess functional diversity. The genetic determinants responsible for many microbial processes have been sequenced[10], being possible to design primers to track homologous sequences in complex samples. Thus, approaches similar to the ones described above for the analysis o 16S rRNA genes can be applied to functional genes. In this way, the molecular diversity of such genes within an environment can be characterised.

A number of studies have been already performed based on such approaches. For example, Felske and co-workers (2003) developed a cultivation-independent functional community profiling method based on PCR-TGGE to study the diversity of mercuric reductase genes and monitor changes in the functional diversity of mercury-reducing biofilm communities. Also, similar approaches but based on PCR-DGGE were applied to unravel the diversity of a photosynthesis-specific gene (*pufM*) (Karr *et al*., 2003) and of a gene coding for the α subunit of ammonia monooxygenase (*amoA*) (Avrahami *et al*., 2003), within complex environmental microbial communities. Alternatively to the study of DNA encoding sequences the detection and analysis of specific mRNAs may be useful to assess gene expression and gene expression fluctuations in response to environmental stresses (Nercessian *et al*., 2005).

Also, oligonucleotide probes targeting environmental functional genes could be used to construct arrays to study molecular diversity of genes or gene expression in the environment (Gibson, 2002). The arrays consist of spatial ordered unique DNA probes matching, for example, sequences encoding different variations of a single enzyme or sequences encoding different enzymes. Total DNA or mRNA extracted from an environmental sample will hybridise with the oligonucleotides on the array if there is a close sequence match (Dennis *et al*., 2003). When using DNA the presence of the target genes will be determined and when using mRNA the expression of those genes will be assessed. Several studies have been conducted using this technique allowing for example

[10] In August 2005, the International Nucleotide Sequence Databases Collaboration announced the world DNA sequence database exceeded 100 gigabases (http://www.ncbi.nlm.nih.gov/Web/Newsltr/V14N2/100gig .html).

the determination of diversity of functional genes in the nitrogen cycle (Taroncher-Oldenburg *et al*., 2003) and the diversity within genes involved in biodegradation and biotransformation (Rhee *et al*., 2004).

Additionally, it has also become possible to study functional diversity by directly cloning and sequencing the environmental DNA from natural communities (metagenome[11]) (Riesenfeld *et al*., 2004b). This approach has allowed studying the diversity of several target genes and even the discovery of a wide range of novel genes and novel activities (Ferrer *et al*., 2005; Kalyuzhnaya *et al*., 2005). The recent advances in sequencing technology have facilitated the development of metagenome-based studies. For example Venter and co-workers (2004) have reported the sequencing of over 1 million kilobases from metagenome libraries from the Sargasso Sea. Also Gill and co-workers (2006) analysed 78 million base pairs of unique DNA sequence from the "microbiome"[12] of two healthy adults.

Finally, a number of culture-independent strategies have been designed in order to link functional and phylogenetic diversity within microbial communities. For example, stable isotope probing (SIP) and the combination of fluorescent in situ hybridisation with microautoradiography (FISH-MAR) have enormous potential for determining which components of a microbial community are capable of incorporating particular substrates (Friedrich, 2006; Wagner *et al*., 2006). However, attributing a microbial process, considered in a complex environment, to a specific microorganism or microorganisms is still a very difficult and challenging task.

1.3 Inferring risk potential from phylogenetic and functional diversities

As described above, prokaryotes are essential to maintain life conditions on our planet, and most of the interactions that they establish with humans are mutually beneficial. Only very few prokaryotes represent a risk to human health, but because of

[11] "Metagenomics" describes the functional and sequence-based analysis of the collective microbial genomes contained in an environmental sample (Riesenfeld *et al*., 2004b).

[12] The term "microbiome" refers to the collective genome of the human intestinal microbiota, which contains at least 100 times as many genes as our own genome (Gill *et al*., 2006).

their importance to man the study of these microorganisms has assumed an enormous significance. Increasing attention has been given to microbial communities from natural environments since these communities may constitute important reservoirs of pathogenic microorganisms and also of potentially hazardous genetic material. However, due to the recently revealed impossibility to cultivate most of the microorganisms from complex microbial communities, microbiologists are now aware that these reservoirs remain fundamentally undisclosed.

Most of the culture-independent studies designed for risk assessment relied simply on PCR detection and/or quantification of specific microorganisms or genes. A limited number of recently reported studies highlighted the usefulness of other culture-independent methodologies to study not only the presence but also the diversity of pathogens or pathogenic genetic determinants in complex samples.

For example, Cocolin and coworkers (2002) and Cocolin and Comi (2005) developed PCR-DGGE methodologies to detect and identify *Listeria* sp. and *Yersinia* sp. respectively, in food samples. Authors stated that the developed method could be considered an important, reliable, and fast method to profile foodborne pathogens and to study their ecology in food samples. Also, Schabereiter-Gurtner and co-workers (2003) successfully applied a strategy based on PCR-DGGE to assess the diversity of bacterial pathogens infecting ticks.

Another example of culture-independent analysis for risk assessment was the construction of 16S rDNA libraries to analyse the bacterial populations in sub-gingival plaque samples from individual suffering from periodontitis[13] (Lillo *et al*., 2004). Also in this case authors considered that the applied methodology had the potential to identify associations between bacterial species and specific disease states.

Finally, metagenomic based-approaches were also applied to assess the genetic pool of microbial communities in hospitals that contribute to public health concerns such as antibiotic resistance and nosocomial infections (Riesenfeld *et al*., 2004b).

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[13] Periodonditis is a dental disorder that results from progression of gingivitis, involving inflammation and infection of the ligaments and bones that support the teeth (http://www.nlm.nih.gov/medlineplus/ency/article/001059.htm).

1.3.1 Natural environments as reservoirs of antibiotic resistance

Resistance to commonly used antimicrobial agents is among the recognised prokaryotic hazardous characteristics representing potential risk for human health. Antibiotic resistance dissemination is the reason why infectious diseases are becoming more severe and require longer, more expensive and more difficult treatments (French, 2005).

Extensive research has been conducted on this subject and several conclusions have been drawn. The emergence of antibiotic resistance has been undoubtedly linked to the extensive and, frequently, erroneous use of antibiotics: resistance mechanisms are most widespread in those health units and countries where antibiotic use is particularly intense (Livermore, 2005). Bacteria have developed several different mechanisms to render ineffective the antibiotics used against them. The genes encoding these defence mechanisms are located on the bacterial chromosome but can also be located on mobile genetic elements, which can be exchanged among bacteria of different taxonomic affiliation (Caratolli, 2001). Mechanisms conferring resistance to virtually all known substances have been detected and characterised from Gram-negative and Gram-positive isolates belonging to a wide range of genera (French, 2005). Taken together, results from the referred studies have highlighted the threat that antibiotic resistance constitute to human health, since it may invalidate the use of substances that constitute the only weapon available to combat numerous infectious diseases.

Most of the knowledge about bacterial antibiotic-resistance and resistance-mechanisms was obtained from studies developed on isolates from clinical environments (French, 2005). However, natural environments are nowadays recognised as important reservoirs of antibiotic resistance microorganisms and of resistance genetic determinants (Alonso *et al*., 2001; Kümmerer, 2003). To assess the extent of the potential risk to human and ecological health a better characterisation of these reservoirs is strongly needed.

Large quantities of antibiotics are used widely in the treatment of infectious diseases in human medicine but also as therapeutic, prophylactic and growth promotion agents in veterinary, agriculture and aquaculture practices (Alonso *et al*., 2001; Kümmerer, 2003). Antibiotics are released into the environment through wastewater effluents and agricultural and animal husbandry runoffs as a result of partial metabolism or inappropriate disposal, raising environmental selective pressures. Also resistant bacteria

are released into the environment through the same courses. Ultimately, aquatic environments are the main receptacles for these contaminants (Chee-Sanford *et al*., 2001). These environments are known to facilitate the exchange of genetic material between bacteria, which can result in higher incidences of multiple antibiotic resistant strains (van Elsas and Bailey, 2002). Natural reservoirs of resistance genes may provide a source of transferable genes, easily spread to pathogenic bacteria and on to humans through the food chain. Additionally these contaminants exert a negative effect on resident bacteria putting the ecosystem stability into peril.

The presence of antibiotic resistant bacteria has been documented from soil (Rysz and Alvarez, 2004), domestic sewage (Goñi-Urriza *et al*., 2000), cattle wastewater (Smith *et al*, 2004), ground water (McKeon *et al*., 1995), surface water (Schwartz *et al*., 2003), drinking water (Schwartz *et al*., 2003), rivers (Ash *et al*., 2002; Goñi-Urriza *et al*., 2000), lakes (Chee-Sanford *et al*., 2001), oceans (Cohen & Colwell, 1986) and estuaries (**Chapter 5**; **Chapter 7;** Rosser *et al*., 1999) from different geographical locations. Also high incidences of resistant bacteria have been found in fish farms and the surrounding aquatic environments (Miranda *et al*., 2003; Saavedra *et al*., 2004; Schmidt *et al*., 2000; Schmidt *et al*., 2001). Rather variable rates of antimicrobial resistance have been reported but frequently high resistance levels to tetracycline, ampicillin, nalidixic acid and trimethoprim have been described for environmental strains (**Chapter 7**; Ash *et al*., 2002; Goñi-Urriza *et al*., 2000; Hatha *et al*., 2005). Resistance of a single bacterial isolate to more than one antimicrobial drug was also commonly detected (**Chapter 7**; Ash *et al*., 2002; Goñi-Urriza *et al*., 2000).

Most of the studies on antibiotic resistance in the environment were basically concerned with the collection of phenotypic data; however, a number of studies have been conducted in order to unravel the presence and molecular diversity of resistance genetic determinants among environmental isolates. The majority of these studies applied PCR and sequencing approaches, using primers targeting previously described genes. For example, primers targeting genetic resistance determinants for tetracycline, gentamicin, β-lactams, vancomycin, streptogramin, trimethoprim and methicillin have been described and applied to study the presence and diversity of such genes in environmental isolates (**Chapter 7**; Hayes *et al*., 2005; Schmidt *et al*., 2001; Volkmann *et al*., 2004). Also, several molecular-based studies reported high levels of incidence and diversity of mobile genetic

elements (usually integrons) carrying a variety of antibiotic resistance genes on isolates from aquatic environments (**Chapter 7**; Schmidt *et al*., 2001).

Most of the referred antibiotic resistance research has been and still is confined to the study of cultivable bacterial isolates. However, as stated in section 1.2.1 of this thesis the cultivable isolates represent only a small fraction of the total microbial community. Consequently most of the environmental pool of resistant genes cannot be assessed using culture-dependent approaches. Recently a limited number of studies were conducted using cultivation-independent approaches to unravel the molecular diversity and molecular ecology of resistance genetic determinants in natural environments.

Aminov and co-workers (2001) developed primer sets targeting tetracycline resistance genes to detect and retrieve homologous sequences from environmental samples. The same primers were applied to assess the presence of such genes in waste lagoons and in ground water (Chee Sanford *et al*., 2001). Authors applied a PCR-DGGE approach to study the molecular diversity of the retrieved DNA fragments. Alternatively, Aminov and co-workers (2002) applied a strategy based on the PCR amplification of tetracycline efflux genes from environmental samples, cloning of the resultant amplicons and sequencing analysis of the constructed libraries. These studies demonstrated the applicability of culture-independent approaches to estimate the environmental resistance gene pool and to follow the flux of such genes in the environment.

PCR-based methodologies were also applied to study the molecular diversity of genes conferring resistance to gentamicin, vancomycin, methicillin and β-lactams in natural environments (Heuer *et al*., 2002; Schwartz *et al*., 2003). Also, studies taking advantage of real-time PCR were found useful for the detection and quantification of resistance genes on complex environmental microbial communities (Smith *et al*., 2004; Volkmann *et al*., 2004).

Finally, two studies were reported that used a metagenome-based approach to discover novel antibiotic resistance genetic determinants from the soil (Riesenfeld *et al*., 2004a) and from the oral cavity (Diaz-Torres *et al*., 2003).

1.3.1.1 β-lactams and β-lactamases

β-lactam substances are the most frequently used antibiotics in the treatment of infectious diseases caused by both Gram-negative and Gram-positive pathogens, because of their safety and selectivity (Demain & Elander, 1999). Also β-lactams are one of the most commonly administered classes of antibiotics in veterinary medicine and agriculture for therapeutic and prophylactic reasons. This class includes rather different substances divided into four sub-classes: penicillins, cephalosporins, carbapenems and monobactams.

The extensive use of β-lactams has resulted in the rapid dissemination of bacterial resistance mechanisms to these antibiotics, the most common being the production of β-lactamases. These enzymes render the antibiotic inactive by hydrolytic cleavage of the β-lactam moiety of the drug. All sub-classes of β-lactams can be hydrolysed by multiple members of the β-lactamase family of enzymes, resulting in biologically ineffective compounds. In fact, the numerous β-lactam antibiotics that have been developed during the last decades, in an attempt to circumvent resistance mechanisms, caused instead the selection of more diverse and potentially more efficient enzymes, namely the so called extended spectrum β-lactamases (ESBLs) (Livermore, 1995). The spread of β-lactamase genes has been greatly aggravated by their association to mobile genetic elements, such as plasmids or integrons, which facilitated the fast dissemination of genetic material between bacteria (Weldhagen, 2004).

Many and diverse β-lactamases were identified and characterised from bacterial isolates. Many attempts were made to classify these enzymes. One of the most generally accepted classification scheme is the one first proposed by Ambler (1980) who divides β-lactamases into 4 molecular classes (A to D) based on their primary structure. Classes A, C and D comprise enzymes that have a serine-based hydrolytic mechanism while class B includes enzymes that have a zinc-based mechanism.

Despite the fact that β-lactam resistance has been registered frequently among environmental isolates, only a few attempts were made to identify and characterise β-lactamases from natural environments, which frequently resulted in the characterisation of novel enzymes displaying novel hydrolytic capabilities (Docquier *et al*., 2002; Henriques *et al*., 2004; Poirel *et al*., 2005; Rossolini *et al*., 2001; Saavedra *et al*., 2003;

Simm *et al*., 2001). Schwartz and co-workers applied a PCR-based approach to detect the presence of β-lactamase genes in total DNA extracted from environmental samples. To our knowledge the study here presented (**Chapter 5**) is the first one to apply culture-independent methodologies in order to study the molecular ecology of β-lactamases in natural environments.

2. *Scope of this thesis*

2.1 Main features of estuarine systems

Estuaries are semi-enclosed coastal bodies of water, tidally-influenced, within which sea water mixes with fresh water. These highly productive ecosystems provide habitat for thousands of birds, mammals, fish, plants and other wildlife species. Also, they are home to large, economically important harbours and offer the ideal conditions for the development of recreational activities, professional fishing and the implementation of aquacultures. Estuaries are also known for their capacity to filter and concentrate pollutants originated from rivers, land and the sea. This pollution can have long-term impacts on the geo-morphological, physical, chemical and ultimately, on the biological characteristics of an estuary.

Estuarine bacterioplankton communities are responsible for the turnover of high amounts of organic matter and play crucial roles on the cycling of several nutrient elements and in photosynthesis. These communities develop and evolve under unstable environmental conditions (Ducklow & Carlson, 1992). Pronounced salinity and temperature gradients, as well as inorganic nutrients and organic matter distribution, contribute to produce different patterns of prokaryotic diversity, abundance and metabolic activity (Bouvier & del Giorgio, 2002; Cottrell & Kirchman, 2000). A few studies reported the existence of typical prokaryotic estuarine communities that may consist of selected freshwater and marine components (Crump *et al*., 1999). Strong spatial and temporal compositional changes and replacement of phylotypes in the bacterioplankton communities between freshwater and saltwater portions of the estuaries have also been reported (Covert & Moran, 2001; Crump *et al*., 1999; Murray *et al*., 1996).

2.2 The sampling site: short characterisation of the Ria de Aveiro

Ria de Aveiro is a very shallow (average depth of 1 m) mesotrophic multi-estuarine ecosystem, located on the Northwest coast of Portugal, 45 km long and 10 km large (Figure 2.1). The estuary has an irregular and complex geometry where four main channels can be identified, which are supplied in freshwater by four rivers: the *Antuã* river, the *Vouga* river, the *Boco* river and the *Caster* river. Water circulation in the *Ria de*

Aveiro is essentially driven by tides, which are semi-diurnal. The tidal water exchange with the ocean is about 89 Mm^3 while the average fresh water input during the time equivalent to a tidal cycle is 1.8 Mm^3. Excluding the areas close to the lagoon mouth, the salinity and the temperature vertical profiles have very well-mixed structures.

Ria de Aveiro is highly polluted due to the presence of harbour facilities, aquaculture ponds, industrial plants, diffuse domestic sewage inputs and run-off from agricultural fields. Those contamination inputs caused a significant eutrophication within the estuary. Also an increasing pressure caused by the anthropogenic activities near its margins, namely building and land occupation, has been registered in the last decades.

Despite the higher pollution levels, *Ria de Aveiro* is characterised by its rich biodiversity and has a great economic importance due to the intense human activity in its waters and along its margins. Professional fishing, recreational activities and aquaculture are important activities in the lagoon and the estuary offers good conditions for agricultural development along its borders and for the setting up of a large number of small and medium industries.

The study here presented was conducted along a longitudinal profile, extending across the inner estuary down to the outer segment of the lagoon (*Canal de Ílhavo*). This channel has 15 km in length, contains a water volume of 2.8 Mm^3 at low tide and 9.3 Mm^3 at high tide and it connects to a permanent fresh water stream (*Rio Boco*) at its upper end, which creates a distinct salinity gradient. **S**even sampling sites spaced regularly at 3 km were defined. From North to South, stations were designated N-1 (in the transition to the coastal zone), I-2, I-4, I-6, I-8 (middle-estuary) and I-10 and RB (defined as the mixing zone between the fresh and marine waters) (Figure 2.1).

Previously several studies were conducted in the same channel concerning the abundance and several aspects of the metabolic activity of the resident bacterioplankton communities (see for example Almeida *et al*., 2002a; Almeida *et al*., 2002b; Cunha *et al*., 2000; Cunha *et al*., 2001).

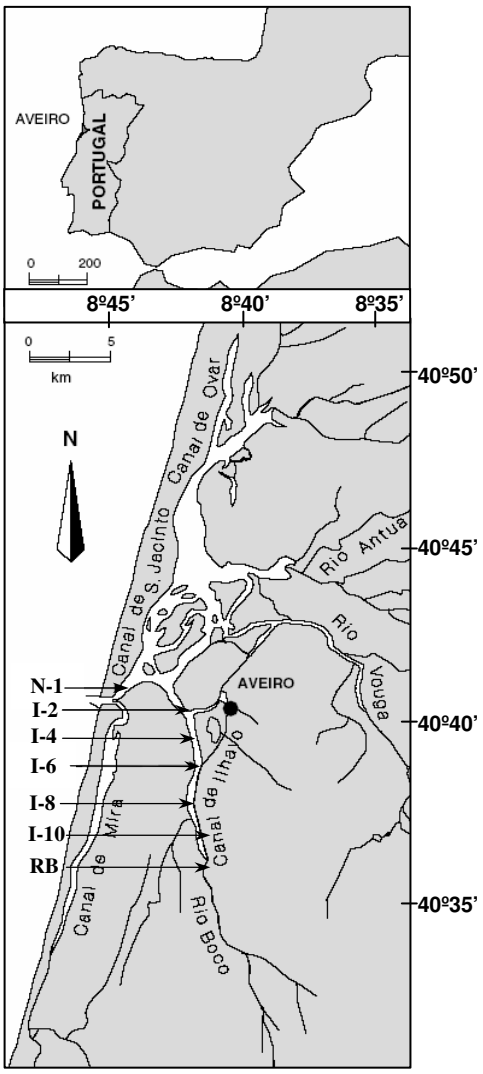

Figure 2.1 *Ria de Aveiro* lagoon with sampling stations indicated by arrows.

2.2 *Objectives of this thesis*

Recent methodological advances highlighted the fact that environmental microbial communities are still fundamentally unknown in what concerns the phylogenetic affiliations as well as the functional and metabolic potential of their members. Additionally, studies are needed in order to understand:

a) to each extent such communities might constitute important reservoirs of potentially hazardous microorganisms and genetic material;
b) how relevant are the exchanges of genetic material between members of the communities in promoting the evolution of pathogenic traits.

The study here presented was developed in order to unravel the phylogenetic diversity of bacterioplankton free-living communities from the estuary *Ria de Aveiro* and also to evaluate the potential of this environment to constitute a reservoir of antibiotic-resistance genetic determinants.

Specifically:

- In **Chapter III** the phylogenetic composition of free-living bacterioplankton populations from sites N-1 and I-6 was investigated through analysis of 16S rRNA genes clone libraries.
- In **Chapter IV** the seasonal and spatial composition dynamics of bacterioplankton communities along the salinity gradient in *Canal de Ílhavo* was analysed using denaturing gradient gel electrophoresis.
- In **Chapter V** culture-independent methodologies were applied to obtain information about the occurrence of DNA sequences putatively encoding β-lactamases in *Ria de Aveiro* and to obtain data on the molecular diversity of those sequences.
- In **Chapter VI** molecular methods were applied in order to characterise the genotypic diversity and phylogenetic affiliation of Gram-negative ampicillin-resistant isolates obtained from *Ria de Aveiro* water samples.
- In **Chapter VII** the aim was to assess the occurrence and molecular diversity of β-lactamase genes among Gram-negative ampicillin-resistant bacteria isolated from

Ria de Aveiro. The presence and diversity of class 1 and class 2 integrons was investigated. In this chapter culture-dependent methodologies were applied.

3. *Molecular sequence analysis of prokaryotic diversity in the middle and outer sections of Ria de Aveiro*

3.1. Introduction

The composition and dynamics of estuarine prokaryotic populations have been studied in a limited number of estuaries (del Giorgio & Bouvier, 2002; Sekigushi *et al*., 2002) and, as stated in the previous chapter, the existence of typical prokaryotic estuarine communities that may consist of selected freshwater and marine components has been reported (Crump *et al*., 1999). Strong compositional changes and replacement of phylotypes in the bacterioplankton communities between freshwater and saltwater portions of the estuaries have also been reported (Covert & Moran, 2001; Crump *et al*., 1999; Murray *et al*., 1996). For a better understanding of the shifts in the composition of prokaryotic populations along the environmental gradients and of the links between composition and metabolic activity, we need much more detailed insights into the phylogenetic affiliation of their most prominent members. In the last decade several molecular methods that avoid the well known limitations of the culture-based techniques have been used to access phylogenetic bacterial diversity in several aquatic environments including estuaries (Bouvier & del Giorgio, 2002; Covert & Moran, 2001; Crump *et al*., 1999).

Generally the *Ria de Aveiro* is dominated by heterotrophic processes (Cunha *et al*., 2000). In a previous study, prokaryotic populations from contrasting sections of the estuary (mid- and outer estuary) were reciprocally exposed to different water; as a result, short-time responses of the rates of ectoenzymatic activity, glucose incorporation and biomass production were displayed (Cunha *et al*., 2001). The unavailability of information on the composition of the communities used in those experiments precluded a complete interpretation of the results obtained.

In this study the phylogenetic composition of free-living bacterioplankton populations was investigated through analysis of clone libraries generated by random cloning of amplified 16S rRNA genes. The molecular analysis reported here revealed a prokaryotic diversity comparable to other coastal and estuarine environments previously studied, but some differences were observed in the types of sequences recovered from each site. This study presents the first characterisation of bacterioplankton communities in *Ria de Aveiro*, even though it assesses only the free-living fraction of the bacterioplankton community.

3.2 Materials and methods

3.2.1 Site description and sample collection

For this study, water samples were obtained from two sampling sites, approximately 9 Km apart, chosen in order to represent contrasting water characteristics within the estuary: station N-1 in *Canal de Navegação*, close to the mouth of the lagoon, representing the deep marine zone, and station I-6 in *Canal de Ílhavo*, representing the shallow inner brackish water zone (see Figure 2.1 – **Chapter 2**). Samples were collected in 2 l autoclaved bottles in May 2002. Water was processed in the laboratory within 1 h after sampling. Salinity was determined with a WTW Conductivity Meter Model LF 196 (WTW, Weinheim, Germany).

3.2.2 Bacterial cell counts

Total bacterial number was determined by direct counting under epifluorescence microscopy (Leitz Laborlux K microscope) with a 50 W mercury lamp, blue BP 450-490 exciter filter and LP 515 barrier filter. The samples were filtered immediately after collection through black 0.2 µm polycarbonate membranes (Poretics Products, Livermore, USA) and stained with 0.03 % acridine orange (Hobbie *et al*., 1977). Images from the microscopic view field were acquired with a DC100 camera (Leica Microsystems, Heildelberg, Germany) and processed with the Leica Qwin image software package (Leica Microsystems, Heildelberg, Germany). For each of the triplicate samples 20 microscope field images were captured (with a total of about 1000 bacterial cells) in order to enumerate the prokaryotic assemblage. All the images were processed with identical image analysis functions.

3.2.3 DNA extraction

For DNA extraction, 450 ml to 3 l water samples were prefiltered through 5-µm polycarbonate filters (Poretics Products, Livermore, USA) and subsequently, bacterial

cells were collected on 0.2-µm filters (Poretics Products, Livermore, USA). Cells were washed from the filters with 2 ml of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and harvested by centrifugation (13000 rpm, 10 min.). The pellet was resuspended in 200 µl TE buffer containing 10 mg/ml of lysozyme, incubated for 1 h at 37 ºC and than frozen and thawed three times. After the described lysis steps, DNA was extracted and purified using the Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania), according to the manufacturer protocol.

3.2.4 Construction of 16S rDNA libraries

Clone libraries were constructed from 16S rDNA sequences amplified from total community DNA. The general bacterial primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') corresponding to positions 8-27 and 1522-1538 on *Escherichia coli* 16S rRNA, were used to amplify nearly full-length 16S rRNA genes (Weisburg *et al*., 1991). PCR was performed in 50 µl reaction mixtures containing 1X PCR buffer (PCR buffer without $MgCl_2$:PCR buffer with $(NH_4)_2SO_4$, 1:1), 3 mM $MgCl_2$, 5 % dimethylsulfoxide, 200 µM each nucleotide, 15 pmol of each primer, 1 U of *Taq* polymerase, and 50-100 ng of purified DNA. The temperature profile was as follows: initial denaturation (94 ºC for 9 min); 30 cycles of denaturation (94 ºC for 30 s), annealing (54 ºC for 30 s), and extension (72 ºC for 90 s); and a final extension (72 ºC for 10 min). The reactions were carried out in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA) using Taq polymerase, nucleotides and buffers purchased from MBI Fermentas (Vilnius, Lithuania).

The resulting amplicons were ligated into the plasmid vector pCR 2.1 by using the TA cloning kit (Invitrogen) according to the manufacturer instructions and transformed into *Escherichia coli* XL2-Blue competent cells (Stratagene, La Jolla, California, USA). For each library, white colonies were randomly picked and screened by PCR for the presence of a complete insert (ca 1500 bp) with primers rD1 and fD1. DNA of each clone was obtained by picking the colony with a sterile toothpick, re-suspension of the cells in 5 µl sterile water and incubation for 10 min at 100 ºC. The lysate so obtained was added to the

PCR mix. The PCR conditions were as described above, except for the annealing temperature that was of 60 ºC in order to avoid amplification of the host 16S rDNA gene.

3.2.5 RFLP analysis and sequencing of cloned 16S rDNA fragments

The inserts from the selected clones were amplified as described and 10 µl of rDNA were digested with 1U of *Hae*III (MBI Fermentas, Vilnius, Lithuania) as recommended by the manufacturer. Products were resolved by electrophoresis in 2.5 % agarose gels at 2.5 V/cm for 3 h using Tris-acetate-EDTA as buffer. The gels were stained with ethidium bromide and the image was digitally processed using a Molecular Image FX apparatus (BioRad, Richmond, USA) and analysed with the Diversity Database software (BioRad, Richmond, USA). Clones showing the same restriction pattern were grouped as discrete operational taxonomic units (OTUs). One or more clones from each OTU were selected for sequencing analysis.

For that, the respective PCR product was purified with the CONCERT™ Rapid PCR Purification System (GIBCO BRL, Eggenstein, Germany) and 5 to 7 µl were used for template in the sequencing reactions. Those were carried out using the primer fD1 and an ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, California, USA). The reaction mixtures were analysed in an automatic DNA sequencer (ABI PRISM® 310 Genetic Analyzer, PE Applied Biosystems). Approximately 550 bp readable sequences were obtained.

3.2.6 Phylogenetic analysis

The CHIMERA_CHECK software (Cole *et al.*, 2003) assessed at the Ribosomal Database Project II (http://rdp.cme.msu.edu/html/) was used to detect PCR-amplified hybrid sequences. Sequences identified as potentially chimeric were not included in subsequent analysis. Clone sequences were compared to the GenBank nucleotide data library using the BLAST software (Altschul *et al.*, 1997) at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) to determine their closest phylogenetic relatives. Sequences were aligned with reference taxa and available

environmental clones within the sequence databases by using the CLUSTAL W program (Higgins *et al.*, 1994) at the European Molecular Biology Laboratory website (http://www.embl-heidelberg.de/). Phylogenetic analyses were performed with PAUP (Swofford, 1999). Trees were produced using the neighbour-joining method based on about 550 nucleotides and rooted using the 16S sequences of *Thermoplasma volcanium* and *Thermoplasma acidophilum* as outgroup. Bootstrap values (1000 replicates) were determined.

As a criterion for refined grouping within an OTU, 97 % similarity was chosen as limit considering that at sequence homology below this value, it is unlikely that organisms are related at the species level (Stackebrandt & Goebel, 1994).

To estimate the representation of the phylotypes, the coverage of each clone library was estimated according to the formula:

$C = [1 - (n1 \times N^{-1})] \times 100\ \%$,

where C is the homologous coverage, n1 the number of OTUs containing only one sequence, and N the total number of 16S rRNA gene clones analysed (Kemp and Aller, 2004).

Accession numbers: The nucleotide sequences determined in the present study have been deposited in the GenBank database under the accession numbers **AY499412**-**AY499468**.

3.3 Results and discussion

3.3.1 Description of sampling sites

Two stations of the *Aveiro* Lagoon were investigated with respect to the phylogenetic composition of their free-living bacterioplankton communities. Station I-6 is less saline than station N-1 which is strongly subjected to marine influence. Salinities were 35.6 and 27.3 for N-1 and I-6 sites respectively. Prokaryotic populations in I-6 water (the less saline site) were 1.8 times denser than the communities from station N-1. Total bacterial number was 3.0×10^9 cell l^{-1} in I-6 water and 1.7×10^9 cell l^{-1} in N-1 water.

3.3.2 Construction and screening of the 16S rDNA libraries

The optimised and applied DNA extraction protocol generated good quality environmental DNA in significant amount, suitable for efficient PCR amplification (Figure 3.1).

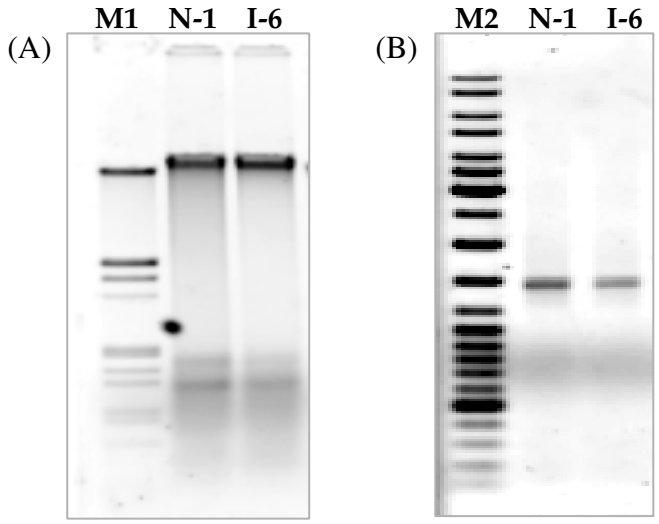

Figure 3.1 (A) Total environmental DNA extracted from sampling sites N-1 and I-6 (2 µl out of 50 µl), loaded on a 0.8 % agarose gel. (B) PCR products obtained from sites N-1 and I-6 using bacterial 16S rDNA specific primers fD1 and rD1 (5 µl out of 50 µl), loaded on a 1 % agarose gel. M1 - DNA size marker λ/*Eco*RI-*Hind*III (MBI Fermentas, Vilnius, Lithuania), M2 - DNA size marker GeneRuler™ DNA ladder mix (MBI Fermentas, Vilnius, Lithuania).

16S rDNA clone libraries were successfully constructed from brackish and marine water samples from the estuary *Ria de Aveiro*. Clones originated from I-6 DNA were named RAI (*Ria de Aveiro* I-6 site) and clones from N-1 were named RAN (*Ria de Aveiro* N-1 site).

A total of 107 RAN clones and 91 RAI clones with approximately 1.5 Kb inserts, randomly selected in respective libraries, were characterised by RFLP analysis using the restriction enzyme *Hae*III (Figure 3.2).

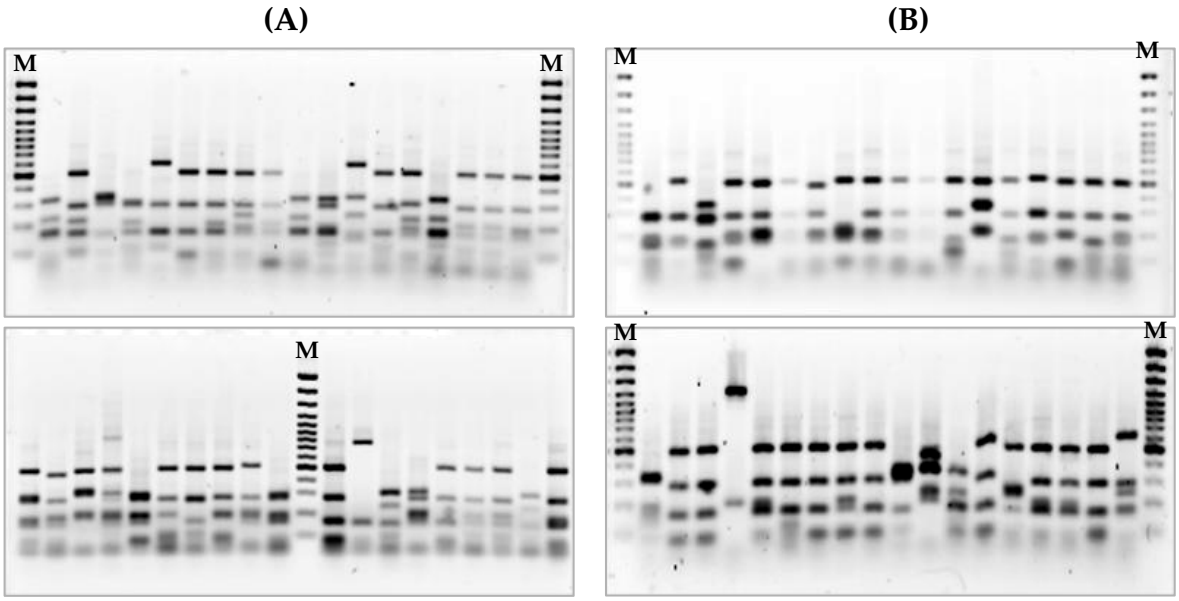

Figure 3.2 Examples of RFLP patterns obtained from clones selected from 16 rDNA libraries constructed from sites I-6 (A) and N-1 (B). M - DNA size marker GeneRuler™ 100 bp DNA ladder plus (MBI Fermentas, Vilnius, Lithuania).

The goal was to sequence representatives from groups of clones with identical RFLP patterns. Based on this data, 63 clones from the I-6 library and 62 clones retrieved from the N-1 library were chosen for sequence analysis. Inserts were successfully sequenced, producing on average 550 bp long sequences, spanning the variable regions V1, V2 and V3; two and three presumed chimeric sequences were detected in the I-6 and N-1 libraries respectively, and were excluded from the analysis. Clones with sequences with more than 97 % similarity were considered to belong to the same operational taxonomic unit (OTU).

Analysis of the nucleotide sequences showed that the sequences obtained from the I-6 and N-1 libraries fell into 25 OTUs and 31 OTUs respectively.

Table 3.1 Phylogenetic affiliation and percent similarity with the closest relative of rRNA gene clones retrieved from N-1 and I-6 libraries. The clone used as representative of each OUT for sequence analysis is marked with an asterisk.

Sampling station	Phylogenetic group	OTUs (sequenced clones)	Closest relative (acession number)	Similarity (%)
Station I-6	*α-Proteobacteria*	**RAI I** (RAI-1*, 78, 90, 122)	Uncultured *Roseobacter* 667-2 (AJ294353)	97
		RAI II (RAI-4*)	Uncultured *α–Proteobacterium* HOC20 (AB054154)	92
		RAI III (RAI-5*, 12, 18, 37, 49, 63, 99, 100, 115)	Uncultured *Roseobacter* NAC11-3 (AF245632)	99
		RAI IV (RAI-9*)	*α–Proteobacterium* OM65 (U70682)	93
		RAI V (RAI-14*)	Uncultured *α–Proteobacterium* 33-FL97B99 (AF469337)	99
		RAI VI (RAI-16, 19, 26*, 66, 81, 82, 84, 114, 118)	Marine *α–Proteobacterium* N67 (AF365990)	99
		RAI VII (RAI-21*)	Marine *α–Proteobacterium* SB11 (AF365998)	97
		RAI VIII (RAI-32*)	*α–Proteobacterium* OM42 (U70680)	98
		RAI IX (RAI-79*)	Marine bacterium Y4I (AF388307)	95
		RAI X (RAI-87, 116*, 125)	*Roseobacter* sp. MED006 (AY136102)	99
		RAI XI (RAI-88, 103*)	Uncultured *α–Proteobacterium* MB11B03 (AY033298)	99
		RAI XII (RAI-94, 101*)	Uncultured proteobacterium OCS14 (AF001643)	97
		RAI XIII (RAI-95*)	Uncultured proteobacterium EBAC46D07 (AF268221)	95
		RAI XIV (RAI-108*)	Uncultured proteobacterium clone BMS32 (AY193223)	97
		RAI .XV (RAI-110*)	Unidentified bacterium (isolate HRV4# HpaAS2) (Z88582)	99
		RAI XVI (RAI-121*)	Marine *α–Proteobacterium* SB11 (AF365998)	97
	β-Proteobacteria	**RAI XVII** (RAI-6, 15, 43*, 54, 83, 92, 96, 113)	*β–Proteobacterium* OM180 (U70707)	97
	γ-Proteobacteria	**RAI XVIII** (RAI-3*, 17, 60, 91)	*Neptunomonas naphthovorans* (AF053734)	95

		RAI XIX (RAI-8*, 77, 93)	Sulfur-oxidising bacterium ODIII-6 (AF170422)	96
		RAI XX (RAI-48*)	*Halomonas* sp. CR1-4 (AY205298)	92
	δ–Proteobacteria	**RAI XXI** (RAI-124*)	*Bdellovibrio* sp. JS7 (AF084862)	89
	Verrucomicrobia	**RAI XXII** (RAI-123*)	Uncultured soil bacterium clone S040 (AY037575)	94
	Bacteroidetes	**RAI XXIII** (RAI-70*)	Uncultured *Bacteroidetes* bacterium clone 1D10 (AY274838)	99
	Actinobacteria	**RAI XXIV** (RAI-76*, 117)	*Actinobacterium* MWH-Dar4 (AJ565416)	97
		RAI XXV (RAI-112*)	*Curtobacterium fangii* (AY273209)	96
Station N-1	*α–Proteobacteria*	**RAN I** (RAN-2*)	*α–Proteobacterium* KAT6 (AF025319)	95
		RAN II (RAN-7, 99*)	Uncultured *α–Proteobacterium* clone CD2H3 (AY038469)	99
		RAN III (RAN-8*)	*Brevundimonas* sp. p22P (AJ495803)	99
		RAN IV (RAN-11, 19, 27, 22, 61*, 71, 75)	Uncultured *α–Proteobacterium* CHAB-I-5 (AJ240910)	99
		RAN V (RAN-12, 14, 79, 104, 127*)	Uncultured *α–Proteobacterium* clone PLY-P3-48 (AY354823)	99
		RAN VI (RAN-13*)	*'Pseudomonas abikonensis'* strain IAM 12404T (AB021416)	98
		RAN VII (RAN-20*)	Uncultured *α–Proteobacterium* CHAB-III-8 (AJ240913)	99
		RAN VIII (RAN-24*, 30 , 51, 140)	Uncultured *α–Proteobacterium* MB13G10 (AY033319)	99
		RAN IX (RAN-57*)	Uncultured *α–Proteobacterium* clone MBAE27 (AJ567586)	96
		RAN X (RAN-63*)	Uncultured *Rhodobacteraceae* bacterium clone RCA11-2 (AY165492)	99
		RAN XI (RAN-72*)	Uncultured proteobacterium OCS124 (AF001644)	99
		RAN XII (RAN-81*)	Marine *α–Proteobacterium* SB11 (AF365998)	97
		RAN XIII (RAN-83*)	Uncultured *α–Proteobacterium* MB11B03 (AY033298)	100
		RXIV (RAN-88*)	Uncultured *α–Proteobacterium* clone CD4C7 (AY038531)	91
		R XV (RAN-98*)	*Roseobacter* sp. MED006 (AY136102)	99

β–Proteobacteria	**RAN XVI** (RAN-5, 29, 41, 80, 115, 133*)	Unidentified bacterium oxSCC-4 (AJ387862)	99
	RAN XVII (RAN-52, 84, 135*)	Uncultured bacterium SJA-10 (AJ009452)	91
	RAN XVIII (RAN-108*)	*β–Proteobacterium* OM180 (U70707)	99
	RAN XIX (RAN-122*)	Uncultured *β–Proteobacterium* clone PLY-P2-82 (AY354843)	99
γ–Proteobacteria	**RAN XX** (RAN-16, 36*)	Uncultured *γ–Proteobacterium* KTc1119 (AF235120)	99
	RAN XXI (RAN-17*, 65)	Uncultured bacterium OHKB4.92 (AB094860)	95
	RAN XXII (RAN-21*, 136)	*γ–Proteobacterium* OM60 (U70696)	99
	RAN XXIII (RAN-53, 56*)	Uncultured marine bacterium ZD0405 (AJ400348)	99
	RAN XXIV (RAN-59*)	*Pseudoalteromonas tetraodonis* IAM 14160T (X82139)	99
	RAN XXV (RAN-60*)	*Pseudomonas pseudoalcaligenes* KF710 (AB109888)	100
	RAN XXVI (RAN-100*)	Uncultured *γ–Proteobacterium* MB12G02 (AY033328)	98
	RAN XXVII (RAN-105*)	Sulfur-oxidising bacterium ODIII6 (AF170422)	96
	RAN XXVIII (RAN-106*)	*Vibrio penaeicida* DSM 14398T (AJ421444)	99
	RAN XXIX (RAN-109*)	Uncultured proteobacterium EBAC31A08 (AF268219)	99
Firmicutes	**RAN XXX** (RAN-18*)	*Bacillus* sp. PL-12 (AF326366)	99
	RAN XXXI (RAN-54, 82, 113, 150*)	*Bacillus* sp. YY (AF414443)	98

3.3.3 Phylogenetic analysis of bacterial 16S rDNA sequences

The coverage of each library was estimated to be 83.5 % and 80.4 % for the I-6 and N-1 sampling sites, respectively. The phylogenetic affiliation of the nucleotide sequences represented in each library is summarised in Table 3.1. The classification of the representatives of the domain *Bacteria* into Phyla or Class was tried by phylogenetic analysis. The closest relative for each sequence was found by BLAST searches against the GenBank database. Most of the sequences were closely related to sequences of uncultured bacterial clones. The sequences of representative clones from each OTU were used to construct Phylum- or Class-specific trees (Figure 3.3).

Both libraries were clearly dominated by clones from the *α–Proteobacteria* (64 % of the total OTUs in the I-6 library and 48 % of the total OTUs in the N-1 library) followed by *γ–Proteobacteria* (12 % of the total OTUs in the I-6 library and 32 % of the total OTUs in the N-1 library). Clones closely related to the *β–Proteobacteria* were also found in both libraries (4 % of the total OTUs in the I-6 library and 13 % of the total OTUs in the N-1 library). Several sequences from those major groups retrieved from both stations fell into the same clusters (Figure 3.3). Additionally, 4 α–*Proteobacteria* OTUs, 1 *β–Proteobacteria* OTU and 1 *γ–Proteobacteria* OTU retrieved from station I-6 were also found in station N-1 (similarity percent > 99 %).

On the other hand, clones affiliated with the *δ–Proteobacteria* (4 % of the total OTUs), *Verrucomicrobia* (4 % of the total OTUs), *Bacteroidetes* (4 % of the total OTUs) and *Actinobacteria* (8 % of the total OTUs) were only found in station I-6 and sequences related to the *Firmicutes* (7 % of the total OTUs) were only found in station N-1.

Among the 31 OTUs affiliated with the *α–Proteobacteria*, 11 OTUs from the I-6 library and 8 OTUs from the N-1 library included close relatives of the *Roseobacter* lineage. Three OTUs from each library were found to be affiliated with the SAR116 clade. Surprisingly, the SAR11 clade, a group routinely recovered in marine clone libraries, was only represented by the clone RAI-14, retrieved from station I-6. This can either be a special characteristic of the studied environment or can be the result of biases associated with the applied methods. However the methods here described were applied by several other authors and the under-representation of the SAR11 group was never, to our knowledge, reported. One of the most referred reason for the under-representation of a

phylogenetic group in a 16S rDNA library is the use of "universal" bacterial primers with mismatches with 16S rRNA genes in that group. However, this is not the case of the primer set used in this study in relation with the SAR11 members (Covert & Moran, 2001). The relative absence of SAR11 relatives in *Ria de Aveiro* estuary needs to be further elucidated. Two clones from the I-6 library and one clone from the N-1 library were closely related to OCS14 and OCS124 environmental clones retrieved from the Oregon coast, USA (Rappé *et al*., 2000). Clone RAN-13 was found to be affiliated with an isolate designated in the GenBank database as '*Pseudomonas abikonensis*' strain IAM 12404T (AB021416). However Anzai and coworkers have shown that this organism is misnamed and that it is rather very close to the genera *Sphyngomonas* (Anzai *et al*., 2000).

From the 13 OTUs affiliated with *γ–Proteobacteria* 10 were retrieved from the N-1 library. Two OTUs from this library were affiliated with the marine clade SAR86. Several N-1 OTUs were closely related to cultured bacterial species namely RAN-60 (100 % similar to *Pseudomonas pseudoalcaligenes*), RAN-59 (99 % similar to *Pseudoalteromonas tetraodonis*), and RAN-106 (99 % similar to *Vibrio panaceia*). Among the *γ–Proteobacteria* clones retrieved from I-6 library RAI-48 was only 92 % similar to *Halomonas* sp. and 4 clones included in OTU RAI XVIII were 95 % similar to *Neptunomonas naphthovorans*.

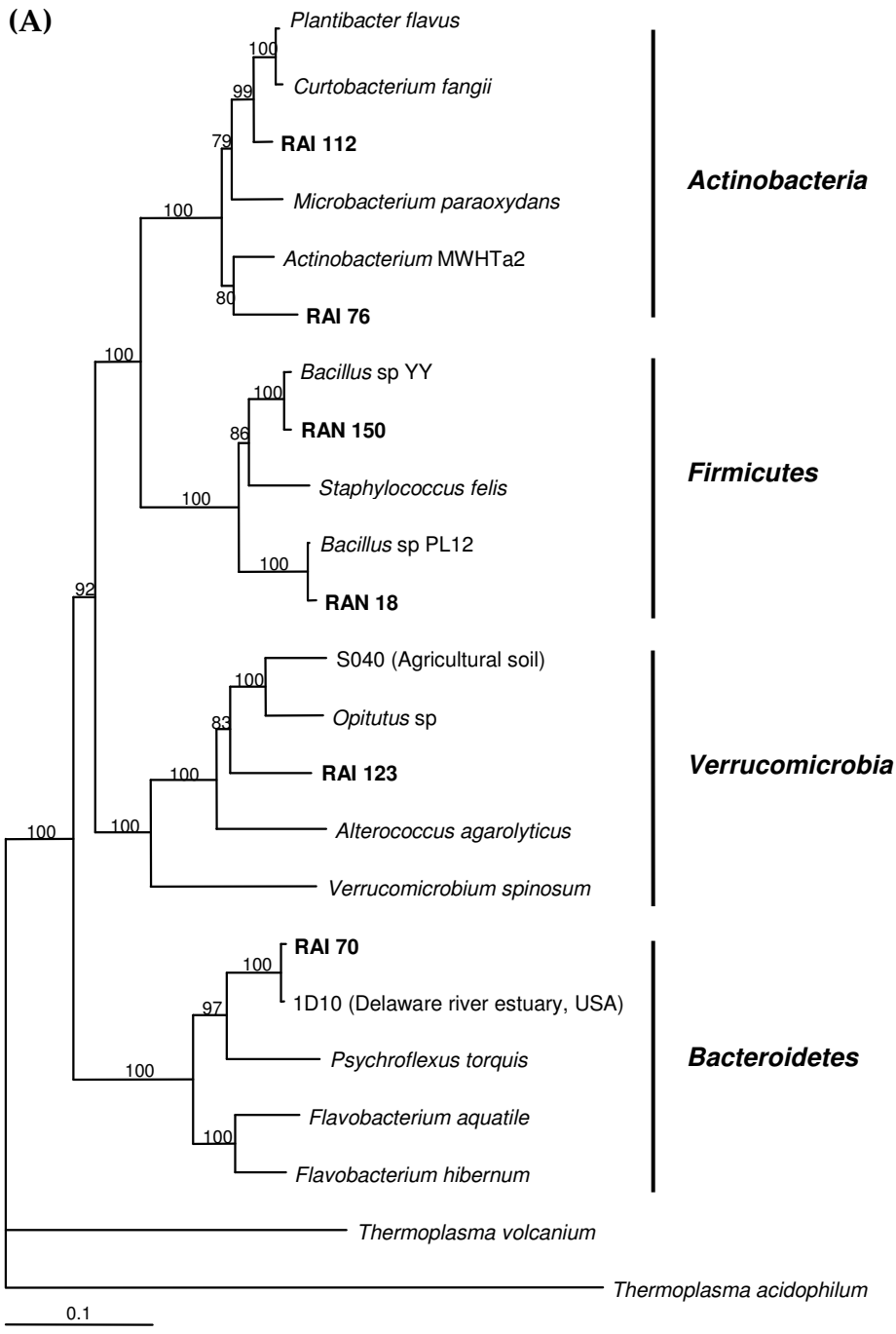

Figure 3.3 Phylogenetic relationships between environmental sequences retrieved from station N-1 and station I-6 bacterioplankton samples (indicated in bold type), reference taxa and available environmental clones: (A) *Actinobacteria, Firmicutes, Verrucomicrobia* and *Bacteroidetes* (B) *α-Proteobacteria* (C) *β-*, *δ-* and *γ- Proteobacteria*. The trees were constructed based on 550 bp. The 16S rRNA gene sequences of *Thermoplasma volcanium and Thermoplasma acidophilum* were used as outgroup. Bootstrap support values (1000 replicates) above 50 % are shown at nodes.

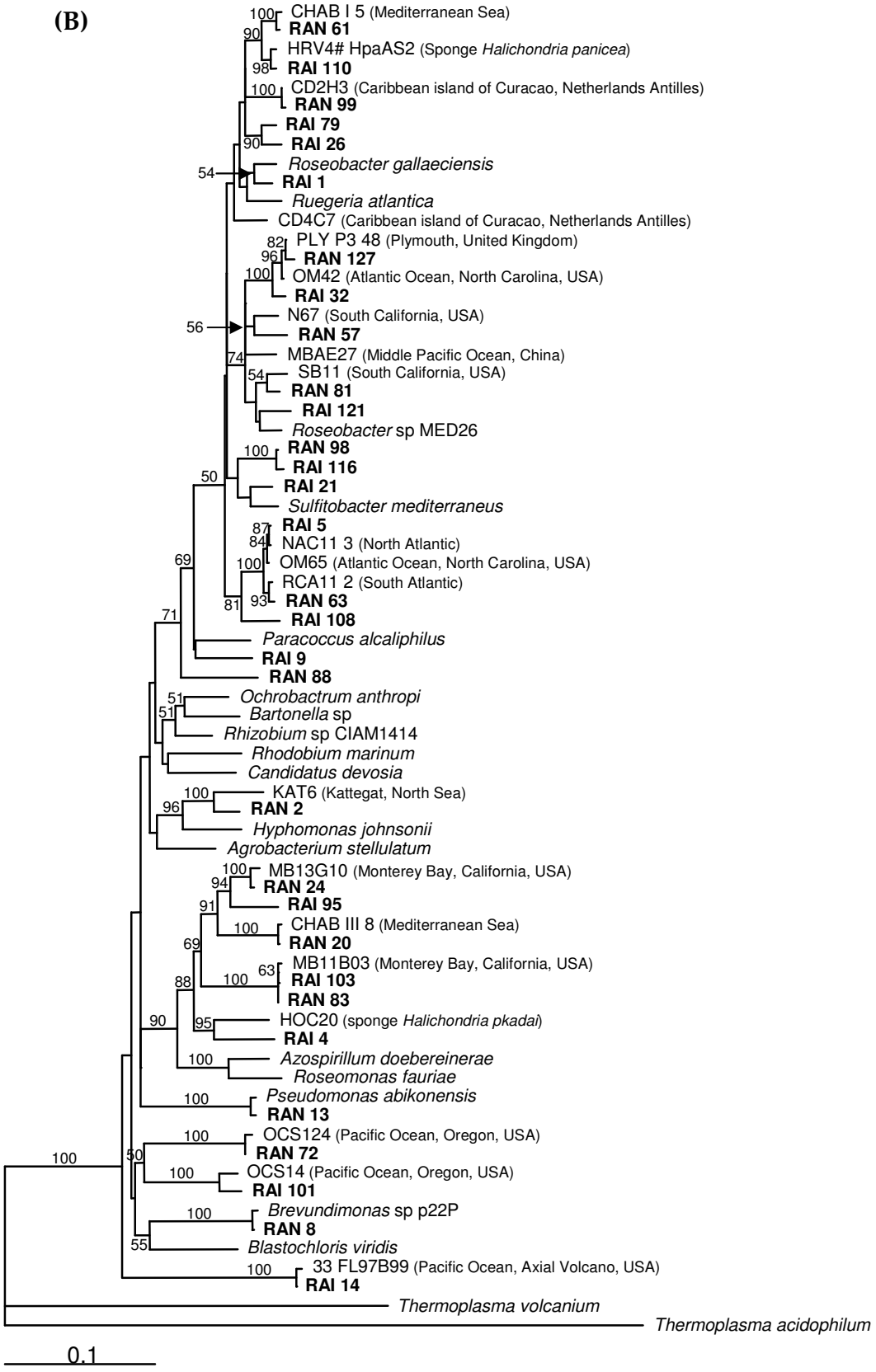

Figure 3.3 Continued

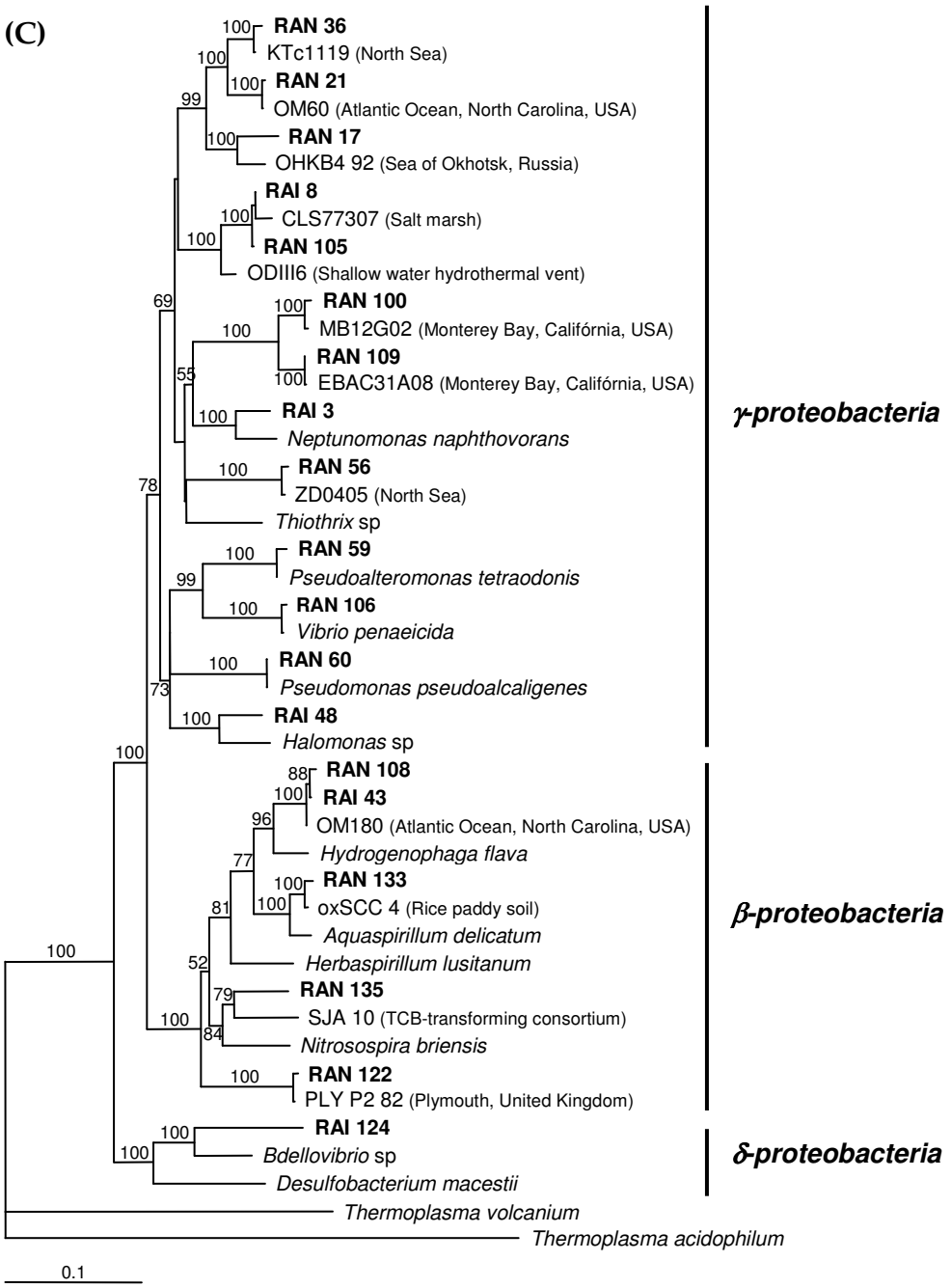

Figure 3.3 Continued

One OTU from I-6 library and 3 OTUs from N-1 library were affiliated to *β-Proteobacteria* members. RAN-108 and 8 clones included in OTU RAI XVII were found to be close relatives of the environmental clone OM180 (>97 % similarity) retrieved from the North Carolina coast, USA (Rappé *et al*., 1997).

Only one clone (RAI-70), retrieved from the I-6 library, was found to be affiliated with the CFB (*Cytophaga-Flexibacter-Bacteroides*) group. However, several studies using fluorescent in situ hybridisation techniques have shown that CFB members were very abundant or even dominant in estuarine and marine bacterioplankton communities (Bouvier and del Giorgio, 2002; Selje and Simon, 2003). Clone libraries of 16S rRNA genes amplified using general bacterial primers probably underestimate the CFB group. Strong mismatches of CFB 16S genes with primer RD1, which might explain the relative absence of the CFB clones in our libraries, have been detected (Glöckner *et al*., 1999). The relative absence of CFB 16S clones in our libraries could also be attributed to the fact that only the free-living bacterioplankton community was assessed, and studies using clone libraries of 16S rRNA genes suggest that this group is enriched in particles but comprises a much smaller fraction of free-living communities (Crump *et al*., 1999).

The clone RAI-124 is quite unique: no sequence very close to it was found in the database (only 89 % similar to *Bdellovibrio* sp.).

Most of the clones found in both libraries were related to clones previously retrieved from estuarine or coastal marine waters. Exceptions were: in the I-6 library one clone affiliated with the *Verrucomicrobia* division (RAI-123) was 94 % similar to a clone retrieved from soil in Georgia, USA (Furlong *et al*., 2002), 4 clones included in OTU RAI XVIII were closely related to *Neptunomonas naphtovorans* isolated from contaminated sediments in Eagle Harbor, Washington, USA (Hedlund *et al*., 1999) and two clones (RAI-76 and RAI-117) were affiliated with *Actinobacterium* MWH-Dar4 retrieved from freshwater in Tanzania (GenBank #AJ565416); additionally clone RAI-112 was closely related to *Curtobacterium fangii* (96 % similarity), a known endophitic bacteria (GenBank #AY273209); in the N-1 library 6 *β–Proteobacteria* clones, included in RAN XVI, were closely related to a sequence retrieved from soils in Italy (Lüdemann *et al*., 2000), 2 clones affiliated with the *γ–Proteobacteria* (RAN-17 and RAN-65) were 95 % similar to a sequence retrieved from marine sediments in Japan (GenBank #AB094860), RAN-18 was closely related to a *Bacillus* strain isolated from Mission Bay sediments, California, USA (Francis

and Tebo, 2002) and 4 clones included in OTU RAN XXXI were found to be a close relative of a *Bacillus* strain isolated from paper mill (Oppong *et al*., 2003).

The general distribution of clones in the two libraries is very similar to that of other clone libraries from coastal and estuarine zones previously characterised (Kelly & Chistoserdov, 2001; Rappé *et al*., 2000; Sekigushi *et al*., 2002*)* even though the composition of the prokaryotic populations from I-6 and N-1 stations differed from one another. The occurrence and even dominance of the *α-Proteobacteria* was already reported in estuarine regions at salinities ranging from 8 to 35 (Bouvier & del Giorgio, 2002; González & Moran, 1997; Sekigushi *et al*., 2002). Bouvier and del Georgio reported that the *α-Proteobacteria* dominated the free-living communities in the lower salt estuary in Choptank river and Pocomoke river estuaries (Bouvier & del Giorgio, 2002). This seems to be also the case of the *Aveiro* lagoon. Within their range of tolerance to salinity, the *α-Proteobacteria* may thus have a competitive advantage at low nutrient concentrations. Cottrell and Kirchman assessed the importance of various phylogenetic groups to bacterial production and demonstrated that α-*Proteobacteria* were the most active bacteria in high salinity locals at the Delaware estuary (Cottrell & Kirchman, 2003). Salinity is undoubtedly an essential factor that affects the composition of microbial communities in the estuaries (Kelly and Chistoserdov, 2001).

The presence of *γ-Proteobacteria* is a common feature of marine and coastal 16S rDNA libraries. Many *γ-Proteobacteria* prefer high nutrient concentration and may be viewed as an opportunist group that takes advantage of transient nutrient enriched conditions.

Actinobacteria and *Verrucomicrobia* were only found at the less saline site (I-6). Both groups are not common in the ocean, thus the abundance of these two phylogenetic divisions may be a general feature distinguishing communities more influenced by freshwater bacterioplankton from its marine counterpart (Stepanauskas *et al*., 2003).

Differences in the metabolic capabilities between bacterioplankton populations from N-1 and I-6 sites have been reported. Cunha and co-workers (2001) reported that bacterial communities in I-6 water were 1.3 to 1.8 denser and up to 10.8 times more active than the communities from the N-1 station. Additionally, previous results of microcosms experiments have shown that samples from the marine station (N-1) exposed to water collected at the inner station (I-6) responded by increasing the total and specific activity rates. However, when after 2 h the test samples were transferred back to the marine

incubation conditions, there was a strong reversion of the responses. Samples from the estuarine station I-6 exposed to marine water incubation were negatively affected and when returned to the estuarine water tank, most of the activity variables did not recover from the negative effect of exposure to marine water (Cunha *et al*., 2001). Activities were measured in the total prokaryotic population (Cunha *et al*., 2001).

As stated by other authors, the particle-attached community is responsible for an important fraction of the metabolic activity in estuaries, and the phylogenetic composition of the free-living community and the particle-attached community is very different (Crump *et al*., 1999; Selje & Simon, 2003). In *Ria de Aveiro* the fraction of particle-attached bacteria in total abundance was reported to be about 19 % on average (Almeida *et al*., 2002b).

This study presents the first characterisation of the prokaryotic assemblage in *Ria de Aveiro*. However the results obtained are not sufficient to explain metabolic activity differences along the estuary since they refer only to the free-living fraction of the bacterioplankton community. Studies including the total prokaryotic assemblage are needed in order to advance from descriptive analysis to quantitative methods focusing on linking particular bacterioplankton with processes. Even though, the information obtained from this study provides an important and valuable base for the design and development of those studies and also for evaluating the effects of environmental fluctuations in this area.

4. Seasonal and spatial variability of free-living bacterial community composition along an estuarine gradient

4.1 Introduction

As stated before, estuaries are usually characterised by strong environmental gradients resulting from the mixing of fresh and marine waters. Salinity, nutrient concentration and organic matter composition gradients are known to strongly influence the composition and structure of the prokaryotic assemblages (Bouvier & del Giorgio, 2002; Ducklow & Carlson, 1992). Those gradients, as well as the mixing of freshwater and seawater communities and the probable existence of a typical prokaryotic estuarine community, are known to create a high degree of bacterial diversity and considerable spatial and temporal variability (Crump *et al*., 2004; del Giorgio & Bouvier, 2002).

As already referred, culture-dependent methods are known to be inadequate for analysis of microbial community because only a small fraction of bacteria in environmental samples are culturable (Ward *et al*., 1990). Molecular methods based on 16S rRNA genes are now widely used for providing new insights into microbial diversity and into structure and dynamics of microbial communities. Particularly, fingerprinting methods such as DGGE (Denaturing Gradient Gel Electrophoresis) of PCR amplified 16S rDNA fragments have been found suitable to analyse multiple samples simultaneously and to access temporal and spatial dynamics of microbial community (Muyzer *et al*., 1993). DGGE analysis has been used to characterise bacterioplankton community succession along several geographically diverse estuaries, such as the Rhone River estuary (Trousselier *et al*., 2002), the Weser estuary (Selje & Simon, 2003), the Plum Island Sound estuarine system (Crump *et al*., 2004) and the Delaware estuary (Castle & Kirchman, 2004). Those studies gave important insights into the composition and variability of bacterioplankton community along the estuarine gradients, namely the confirmation that salinity plays a key role in determining the bacterioplankton distribution.

Previously, *Ria de Aveiro* has been extensively characterised in what concerns physical and chemical parameters like temperature, salinity, available nutrients and dissolved oxygen (Almeida *et al*., 2002a; Cunha *et al*., 2000; Lopes *et al*., 2005). Along the salinity gradient in *Ria de Aveiro*, bacterioplankton populations displaying different metabolic capabilities have been reported. However, whether these differences are due to metabolic plasticity or to genetically diverse microbial assemblages was not elucidated, since data on their phylogenetic composition was not available (Almeida *et al*., 2002a; Cunha *et al*., 2000; Cunha *et al*., 2001).

Previously we used 16S rDNA clone libraries to examine the free-living bacterial community structure within the middle and outer sections of *Ria de Aveiro* (**Chapter 3**). Results obtained revealed a prokaryotic diversity comparable to other coastal and estuarine environments previously studied. On the other hand, some differences were observed in the types of sequences recovered from the two sections of the estuary. However, the preceding study included only two sampling sites and didn't assess the temporal variability.

The aim of this study was to examine the spatial and temporal distribution of the *Bacteria* phylotypes present in the free-living bacterial community in the estuarine system *Ria de Aveiro*. For that we used DGGE analysis of PCR amplified fragments of the 16S rDNA. The contribution of salinity and temperature on bacterioplankton community distribution was assessed using CCA (Canonical Correspondence Analysis). The identity of the predominant community members was assessed by analysis of sequences retrieved from DGGE-separated DNA fragments and comparison with sequences stored in databases.

4.2 Materials and methods

4.2.1 Site description and sampling

Samples were collected in 2 L autoclaved dark bottles always during daytime, at low tide, approximately 0.2 m below the surface, in April (spring), July (summer), and October (autumn) 2003 and in January (winter) 2004. Seven sampling sites spaced regularly at 3 km were defined. From North to South, stations were designated N-1 (in the transition to the coastal zone), I-2, I-4, I-6, I-8 (middle-estuary) and I-10 and RB (defined as the mixing zone between the fresh and marine waters) (see Figure 2.1 – **Chapter 2**). To simplify the presentation of results, subsequently station N-1 will be referred as the marine section of the estuary, sites I-2, I-4, I-6 and I-8 as the brackish section and sites I-10 and RB as the freshwater section. Water was processed in the laboratory within 1 h after sampling. Salinity was determined with a WTW Conductivity Meter Model LF 196 (WTW, Weinheim, Germany).

4.2.2 DNA extraction, PCR and DGGE

Water samples were pre-filtered through 5-µm-pore-size polycarbonate filters (Poretics Products, Livermore, USA) and then bacterial cells were collected on 0.2-µm-pore-size filters (Poretics Products, Livermore, USA). DNA extraction was performed as described in chapter 3. The V3 region of bacterial 16S rDNA fragments was amplified using the primers 338F (5'-GACTCCTACGGGAGGCAGCAG-3') and 518R (5'-ATTACCGCGGCTGCTGG-3') with a GC clamp attached to the forward primer (Muyzer *et al*., 1993). PCR was performed in 50 µl reaction mixtures containing 1X PCR buffer (PCR buffer without $MgCl_2$:PCR buffer with $(NH_4)_2SO_4$, 1:1), 3 mM $MgCl_2$, 5 % dimethylsulfoxide, 200 µM each nucleotide, 15 pmol of each primer, 1 U of *Taq* polymerase, and 50-100 ng of purified DNA. The temperature profile was as follows: initial denaturation (94 ºC for 5 min); 30 cycles of denaturation (92 ºC for 30 s), annealing (55 ºC for 30 s), and extension (72 ºC for 30 s); and a final extension (72 ºC for 7 min). The reactions were carried out in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA) using Taq polymerase, nucleotides and buffers purchased from MBI Fermentas (Vilnius, Lithuania).

DGGE was performed on a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, California, USA). Samples containing approximately equal amounts of PCR amplicons were loaded onto 8 % polyacrylamide gels (37.5:1, acrylamide: bisacrylamide) in 0.5X TAE buffer (20 mM Tris-acetate, pH 7.4, 10 mM sodium acetate, 0.5 mM Na_2EDTA) using a denaturing gradient ranging from 35 % to 50 % (100 % denaturant contains 7 M urea and 40 % formamide). Electrophoresis was performed at 60 ºC, initially at 20 V (15 min) and then at 200 V (330 min). The gels were stained in an ethidium bromide solution (5 min) and then rinsed in distilled water (20 min). The image was acquired using a Molecular Image FX apparatus (Bio-Rad Laboratories, Hercules, California, USA).

4.2.3. *Analysis of DGGE profiles*

Gel images were analysed with the Diversity Database™ Fingerprinting software (Bio-Rad Laboratories, Hercules, California, USA). Every gel contained 3 lanes with a standard of 8 bands for internal and external normalisation and as an indication of the quality of the analysis. Bands with a relative intensity of less than 0.2 % of the sum of all band intensities were discarded. Several of the weaker bands could not be distinguished in the figure presented here (Figure 4.1), and were only detectable after zooming the image. The bands occupying the same position in the different lanes of the gels were identified. A binary (1/0) matrix was constructed taking into account the presence or absence of individual bands in each lane. Cluster analysis and non-metric multidimensional scaling (NMDS) were performed using the PRIMER v5 software (Clarke & Gorley, 2001). The binary matrix was transformed into a similarity matrix using the Bray-Curtis measure. Dendrograms were generated using the group average method.

CCA was used to relate seasonal and spatial succession of genetic fingerprints (binary matrix of DGGE banding profiles) to salinity and temperature values (after standardisation). The statistical significance of the relationship was assessed by Monte Carlo permutations test using 1000 permutations. The analysis was carried out using the software package CANOCO 4.5 for Windows (ter Braak & Verdonschot, 1995).

4.2.4 *Sequencing of DGGE bands and phylogenetic analysis*

DGGE bands were excised with a sterile scalpel and eluted in 20 µl of sterile water, overnight at 4 ºC. Five µl of the supernatant were used for re-amplification with the original primer set. The accuracy of the bands and the position in the gel were checked on DGGE gels together with the original sample. Whenever necessary, bands were processed again as described above.

For sequencing analysis, PCR products were purified with the Concert™ Rapid PCR Purification System (Gibco BRL, Eggenstein, Germany) and used as template in the sequencing reactions. Those were carried out using the primer 518R and an ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems,

Foster City, California, USA). The reaction mixtures were analysed in an automatic DNA sequencer (ABI PRISM® 310 Genetic Analyser, PE Applied Biosystems).

For some bands it was not possible to obtain quality sequences using the above strategy. Therefore, after re-amplification with primers 338F (without the GC clamp) and 518R, the resulting amplicons were cloned using a TA cloning kit (Invitrogen) according to the manufacturer instructions. Subsequently, at least 6 inserts were checked by PCR-DGGE and subjected to sequencing analysis as described above.

Band sequences were compared to the GenBank nucleotide data library using the BLAST software at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) in order to determine their closest phylogenetic relatives. Sequences were aligned with reference taxa and available environmental clones within the sequence databases using the CLUSTAL X program (Thompson *et al*., 1997). Phylogenetic analyses were performed with PAUP* version 4.0b10 (Swofford, 2003). Trees were produced using the neighbour-joining method.

Accession numbers: The nucleotide sequences determined in the present study have been deposited in the GenBank database under the accession numbers **DQ099500**-**DQ099523**.

4.3 Results

4.3.1 DGGE analysis of bacterial community structure

The DGGE profiles obtained for seven sampling sites distributed along the estuary, in April, July and October 2003 and January 2004, are shown in Figure 4.1. Reproducibility of PCR amplification and DGGE was confirmed by three replicates. The profiles were highly reproducible between gels and PCRs. The total number of band positions detected in the four gels was 45 and the number of DGGE bands per sample varied between 17 and 24, being rather stable in all samples. DGGE banding patterns from samples collected in the middle estuary (sites I-2, I-4, I-6 and I-8) shared many bands, many of which were detected in all seasons, indicating the presence of several widespread

phylotypes in this area of the estuary. Identical profiles were obtained from contiguous sites, namely from I-2 and I-4 samples collected in April, from I-4, I-6 and I-8 samples collected in July and from samples I-4 and I-6 collected in January. Also, profiles obtained from samples collected in the inner estuary (sites I-10 and RB) shared many bands.

Cluster analysis was performed to gain an overview on the relatedness of phylogenetic profiles representing the communities at each site and at each sampling date (Figure 4.2). This analysis corroborates the existence of distinct bacterial communities in the marine-brackish sections and in the freshwater section of the estuary. The middle estuarine samples (I-2, I-4 and I-6) clustered mostly according to time of sampling, indicating that the temporal factor was more important than the spatial one in determining this bacterial assemblages composition. Four temporal clusters of samples could be identified corresponding to the four seasons. This analysis also showed that the samples collected from the middle estuary on April and July branched more closely to each other than to the samples collected in October and particularly in January. On the other hand samples collected from the freshwater section of the estuary (I-10 and RB) form two clusters only distantly related to the brackish and marine samples being clearly a result from spatial factors. One of those clusters (samples April RB and I-10, October RB and January RB) includes water samples with salinities ranging from 4 to 19; the other (April I-8, July I-10 and RB, October I-10 and I-8 and January I-10), includes water samples with salinities ranging from 0 to 0.8. Samples collected from site I-8 clustered both with the brackish samples (I-8 collected in July and January) or the freshwater samples (I-8 collected in October and April).

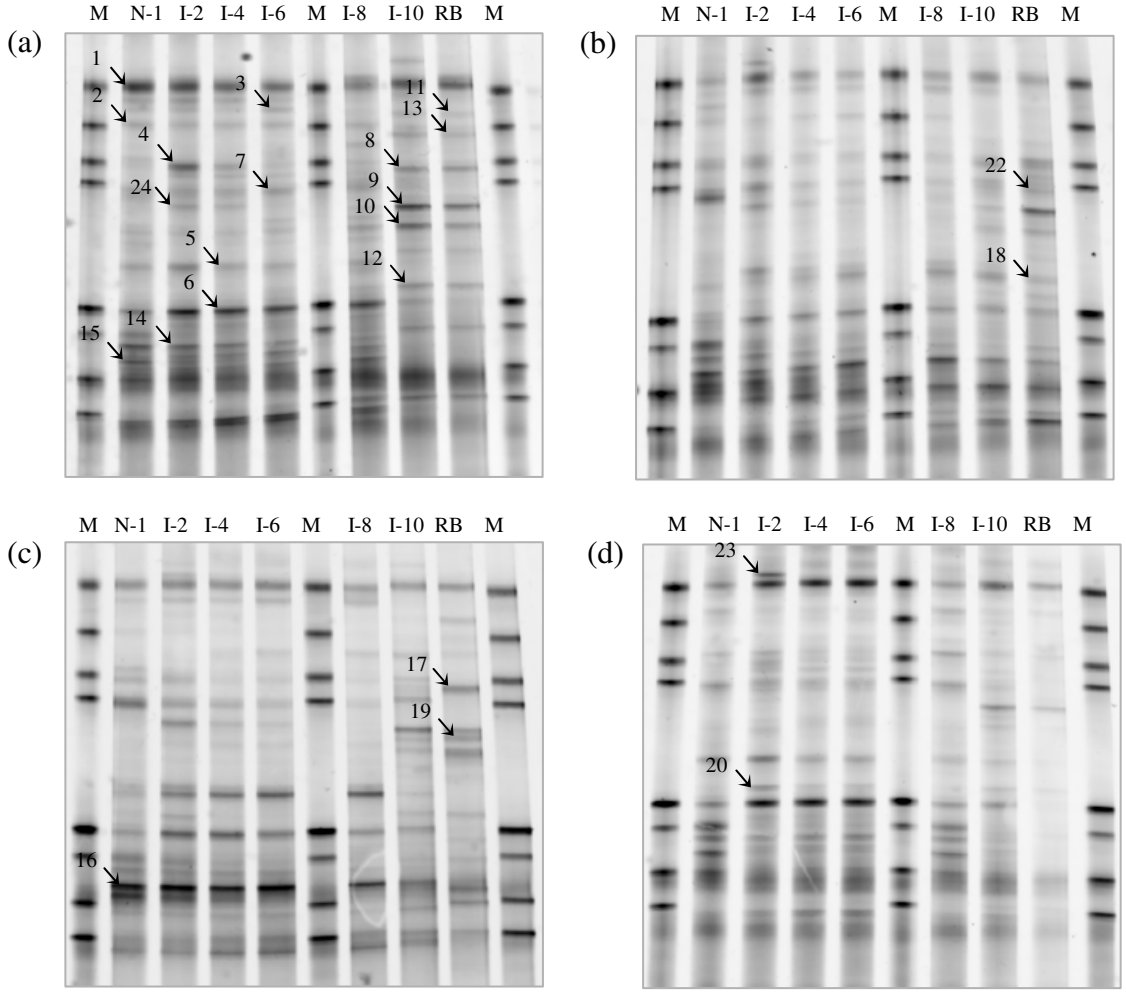

Figure 4.1 DGGE gels showing PCR-amplified bacterial 16S rDNA fragments for the 7 samples investigated (indicated on top of the lanes), collected in April 2003 (a), July 2003 (b), October 2003 (c) and January 2004 (d). Lane M: DGGE marker constructed using previously characterised 16S rDNA clones from environmental libraries (**Chapter 3**). Bands that were excised and cloned for sequence analysis are labelled with the same number as in Table 4.2 and indicated with an arrow.

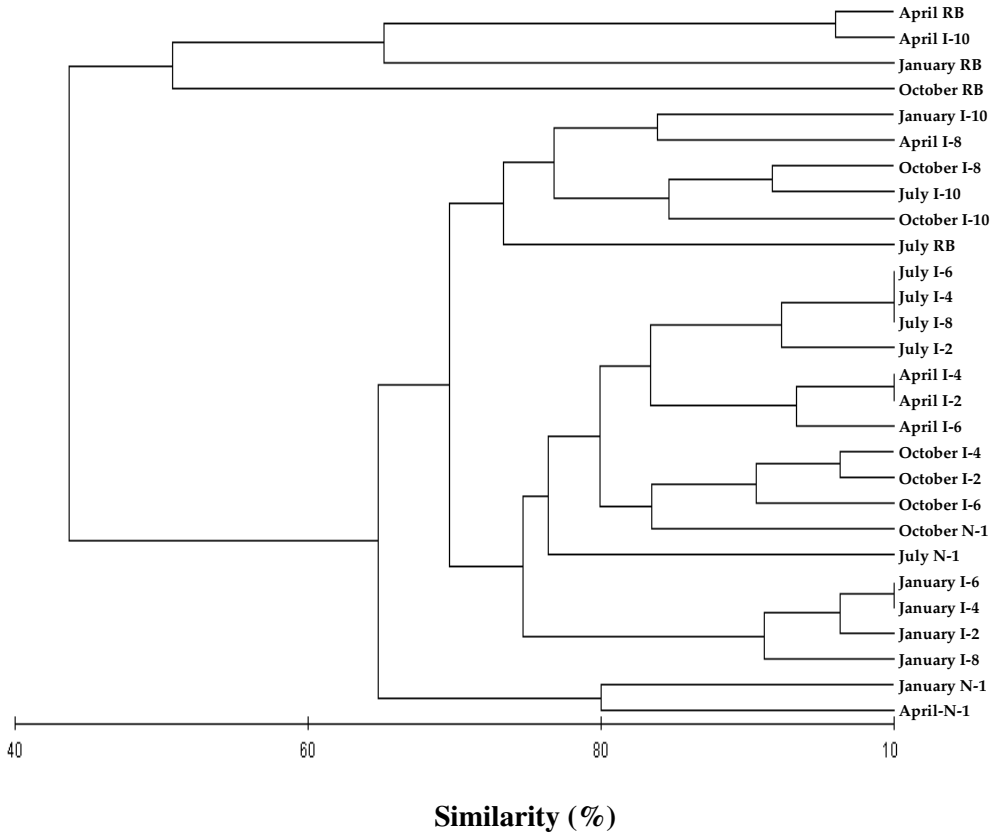

Figure 4.2 Cluster analysis of bacterial communities based upon DGGE profiles. Similarities were calculated using the Bray-Curtis measure.

MDS analysis of the DGGE band profiles (Figure 4.3) showed that the bacterial community gradually and continuously changed from the marine zone (site N-1) to the freshwater section, tending to track salinity. However, some major shifts of the bacterial community structure could be identified: in April between sites I-8 and I-10 and in October and January between samples I-10 and RB. This data-reducing method places each complex DGGE fingerprint as 1 point in a 2D diagram with artificial axis, in a way that more similar samples are plotted closer together.

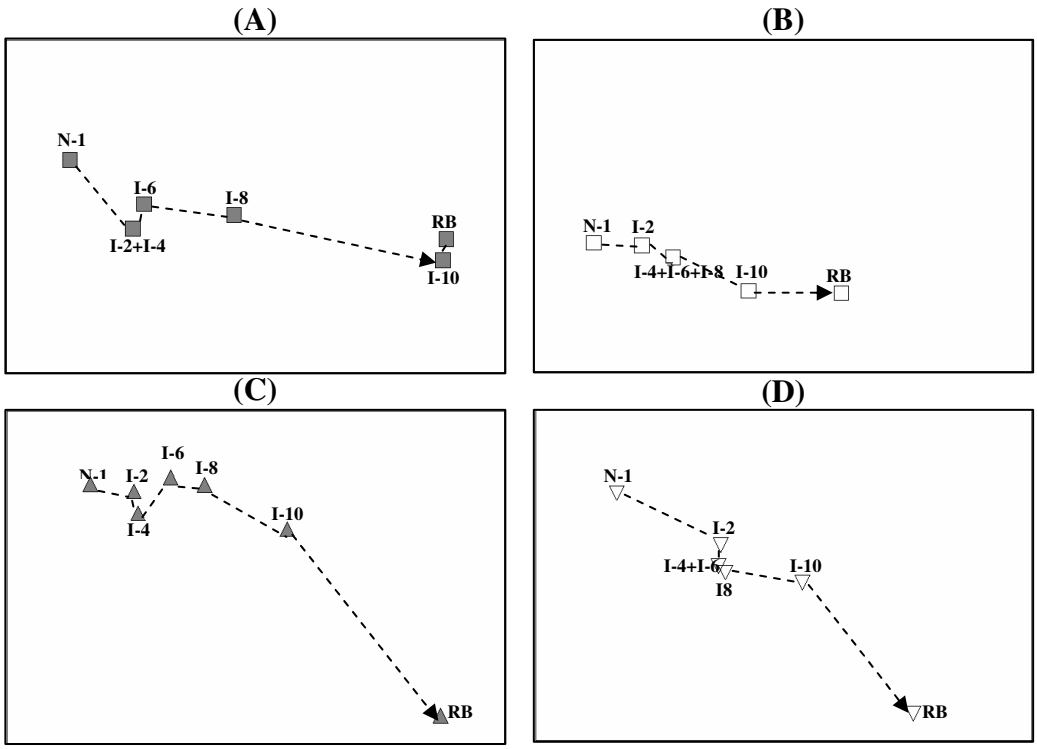

Figure 4.3 Non-metric multidimensional scaling diagrams of similarity matrices calculated from DGGE patters of samples collected in April (A), July (B), October (C) and January (D). Consecutive samples were connected by lines in order to follow the changes of the bacterial assemblage along the sampling gradient. The stress values of the final configurations ranged between 0.06 and 0.069.

CCA of DGGE profiles scaled with salinity and temperature (biplot presented in Figure 4.4) revealed that the axis related to salinity described 19 % of the variability of DGGE patterns and the axis related to temperature describes only 7 % (a Monte Carlo permutation test showed statistical significance $P \leq 0.005$). The unexplained fraction, i.e. the amount of variation that is explained by unknown (non-studied) factors, represents 74 % of the total variation. The seasonal succession of the patterns occurred with small differences between samples from April and October while samples from July and January are clearly separated based on temperature. On the other hand, the salinity parameter clearly separated samples from the inner estuary (I-10 and RB sites) from samples from the brackish and marine zones (N-1, I-2 and I-4 sites). However, samples collected in July from sites I-10 and RB are not so clearly separated from the other samples

collected in this month. This results from the fact that those samples, as shown in Table 4.1, are characterised by unusually high salinity values. CCA of DGGE profiles for each month separately scaled with salinity and temperature accounted for 57 % to 78 % of the variability confirming that those are major factors in determining spatial variability of bacterioplankton communities within the estuary.

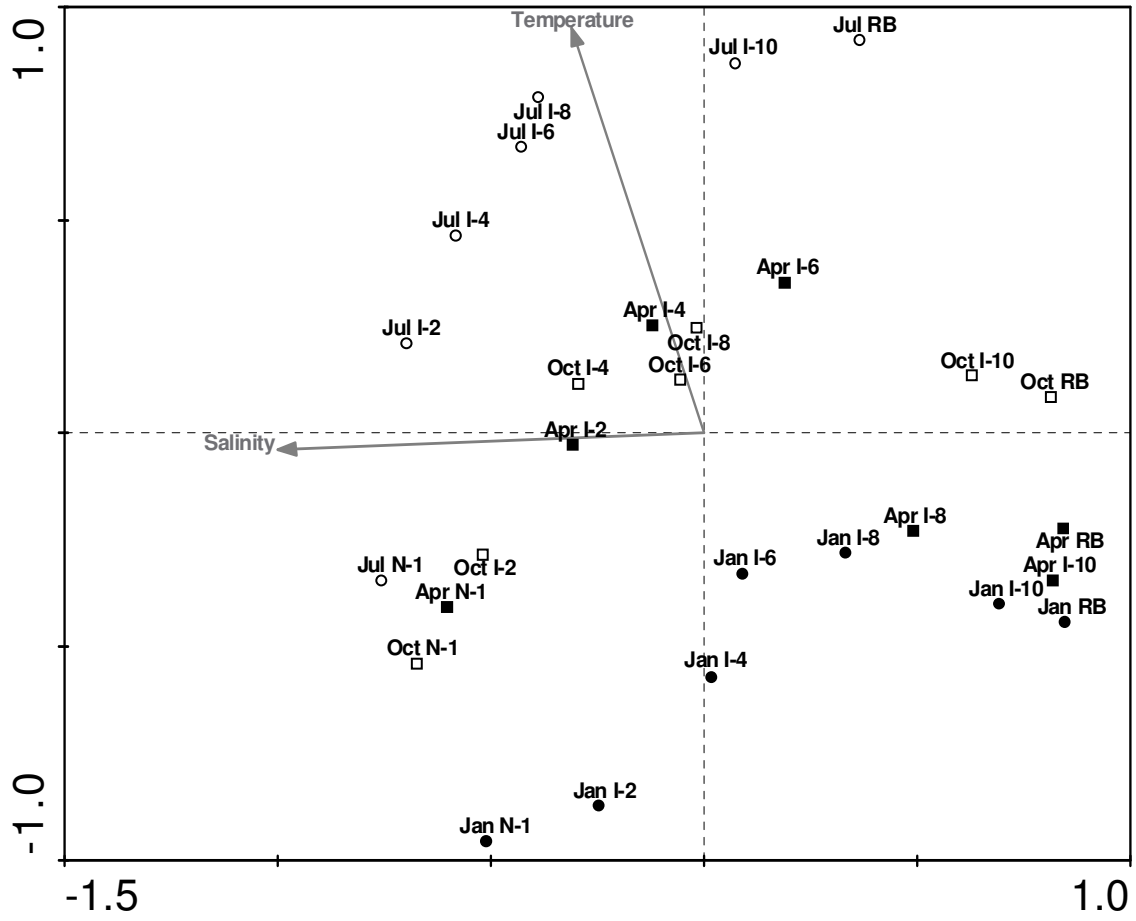

Figure 4.4 CCA ordination biplot of DGGE bands (samples indicated using the month and site of sampling) and environmental variables (represented by arrows). ■ (samples collected in April); ○ (samples collected in July); □ (samples collected in October); ● (samples collected in January).

4.3.2 Sequencing and identification of DGGE fragments

Twenty two bands were excised from gel tracks corresponding to marine, estuarine and freshwater samples, mainly from patterns corresponding to samples collected in spring (Table 4.2). Occasionally, unique bands detected in samples collected in a different season were also excised. Most of the excised bands were directly sequenced; however, bands 3, 8, 11, 12, 15, 18, 19 and 20 in Figure 4.1 were sequenced after cloning. Band 24 was discarded from the analysis: after cloning several inserts were checked but we could not observe a band migrating in the original position in DGGE gels.

Table 4.1 Temperature and salinity profiles from *Ria de Aveiro*.

Site	Temperature (ºC)				Salinity			
	April	July	October	January	April	July	October	January
N-1	16.5	17.0	16.0	14.0	31.4	35.1	33.3	32.0
I-2	17.5	19.0	16.5	14.0	24.1	33.7	29.7	25.5
I-4	18.5	20.0	18.0	15.0	21.6	30.6	23.8	18.6
I-6	18.5	21.0	18.0	16.0	13.6	28.7	20.1	16.6
I-8	16.0	21.0	18.0	16.0	8.9	27.7	19.0	12.9
I-10	15.0	21.0	17.0	15.0	0.8	15.7	5.3	4.0
RB	15.0	21.0	16.5	14.5	0.0	8.4	0.6	0.0

Some bands, e. g. bands 2, 3, 4, 5, 6, 7, 14 and 15, were more pronounced in the marine (N-1) and brackish (I-2, I-4, I-6 and I-8) sections of the estuary, but disappeared in the freshwater section. Others, e. g. bands 8, 9, 10, 11, 12 and 13, occurred only or were more pronounced in the freshwater section (I-10 and RB). A few bands were identified only in the brackish section of the estuary, e. g. bands 20, 23 and 24; however, generally, patterns obtained from station N-1 were similar to the contiguous brackish sites. Band 1 and 6 were found in all samples collected. To confirm that matching bands corresponded to identical phylotypes, bands 1 and 6 were sequenced from stations N-1, I-6 and RB from

samples collected in April, July, October and January. Also bands 9 and 10 from all samples collected from sites I-10 and RB were sequenced. The same nucleotide sequence was obtained for each band position.

Most of the excised bands corresponded to a unique DNA sequence. However bands 12 and 18 were composed by pairs of clearly different DNA sequences (identified as a and b in Table 4.2).

Most of the sequences were similar to 16S rDNA sequences reported from uncultured organisms present in environmental samples from sources such as lakes, rivers, estuaries and coastal waters (Table 4.2; Figure 4.5). According to BLAST results, 7 sequences affiliated with phylum *Bacteroidetes*, 5 with sub-class *α-Proteobacteria*, 4 with *γ-Proteobacteria*, 2 with *β-Proteobacteria*, 2 with *δ-Proteobacteria*, 2 with *ε-Proteobacteria* and 1 sequence was identified as a chloroplast-like sequence. Sequences retrieved from the marine and brackish sections were all affiliated with *Bacteroidetes*, *α-Proteobacteria* or *γ-Proteobacteria*, while most of the sequences retrieved from the freshwater section were affiliated with *β-*, *δ-*, and *ε-Proteobacteria* and also with *Bacteroidetes*. The sequences obtained from each band, except for the chloroplast affiliated sequence, were used to construct Phylum- or Class-specific trees (Figure 4.5).

The most intense bands from marine and brackish sections in April, July and January were bands 1 and 6. Sequence from band 1 was 99 % similar to clone PI_4t10c, retrieved from Plum Island Sound Estuary, USA, affiliated to *Bacteroidetes* (Acinas *et al*., 2004). Band 6 was identified as an *α-Proteobacteria* clone, closely related to *Roseobacter* sp. J504, isolated from a marine sponge (Sfanos *et al*., 2005). In October the most prominent band in those sites was band 16, 98 % similar to *Mesorhizobium* sp. ICMP 12635 isolated from *Carmichaelia petriei* in New Zealand (Weir *et al*., 2004).

Band 1 was also prominent in several samples collected from the inner estuary (samples I-10 and RB). In those samples bands 8, 9 and 10 were also intense. Band 8 was identified as *β-Proteobacteria*, being closely related to clone LS-E5, retrieved from Tillamook Bay, USA (Bernhard *et al*., 2005). Bands 9 and 10 were both *Bacteroidetes* clones, 100 % identical to *Flavobacterium* sp. Man01 isolated from lake Pontchartrain, USA (band 9) and clone NE60, retrieved from lake Grosse Fuchskuhle, Germany (band 10) (Burkert *et al*., 2003).

Bands 20 and 23 were detected only in the brackish section of the estuary. Band 20 was 100 % identical to the 16S rDNA sequence of the uncultured marine *γ-Proteobacteria* bacterium COL-6, retrieved from diatom detritus (Bidle & Azam, 2001); band 23 was identified as γ-*Proteobacteria*, being closely related to clone RAI-3, retrieved from the 16S rDNA library constructed from the I-6 site of *Ria de Aveiro* (**Chapter 3**).

Table 4.2 Phylogenetic affiliation of DGGE band DNA sequences.

Band nº.	Acession nº.	Sample	Closest relative (acession nº)	Origin	Phylogenetic aphiliation	% similarity
B1	DQ099500	N-1-April	Uncultured *Bacteroidetes* bacterium clone PI_4t10c (AY580602)	Plum Island Sound Estuary, USA	*Bacteroidetes*	99
B2	DQ099501	N-1-April	Uncultured *Bacteroidetes* bacterium clone PI_4t2a (AY580618)	Plum Island Sound Estuary, USA	*Bacteroidetes*	99
B3	DQ099502	I-6-April	Uncultured prasinophyte clone LS-C12 (AY628653)	Tillamook Bay, USA	Chloroplast	99
B4	DQ099503	I-2-April	Gamma proteobacterium MED23 (AF025566)	NW Mediterranean	γ-*Proteobacteria*	100
B5	DQ099504	I-4-April	Uncultured gamma proteobacterium clone YC499B15_O (AY701439)	*Gymnodinium catenatum*	γ-*Proteobacteria*	100
B6	DQ099505	I-4-April	*Roseobacter* sp. J504 (AY369979)	Marine sponge, *Porifera*	*α-Proteobacteria*	100
B7	DQ099506	I-6-April	Flavobacteriaceae bacterium G912S3A (AY353822)	Southern Ocean	*Bacteroidetes*	100
B8	DQ099507	I-10-April	Uncultured beta proteobacterium clone LS-E5 (AY628664)	Tillamook Bay, USA	*β-Proteobacteria*	100
B9	DQ099508	I-10-April	Flavobacterium sp. Man01 (AY788963)	Lake Pontchartrain, USA	*Bacteroidetes*	100
B10	DQ099509	I-10-April	Uncultured *Bacteroidetes* clone NE60 (UBA575728)	Lake Grosse Fuchskuhle, Germany	*Bacteroidetes*	100
B11	DQ099510	RB-April	Uncultured *Bacteroidetes* bacterium clone Sta2-21 (AY562331)	Delaware River, USA	*Bacteroidetes*	99
B12a	DQ099511	I-10-April	Aquatic bacterium R1-B24 (AB195756)	Lake Inba, Japan	*β-Proteobacteria*	98
B12b	DQ099512	I-10-April	Saltmarsh clone LCP-26 (AF286031)	Mercury and PCB contaminated sediment	*δ-Proteobacteria*	91

B13	DQ099513	RB-April	Uncultured *Bacteroidetes* bacterium clone Sta2-97 (AY562365)	Delaware River, USA	*Bacteroidetes*	98
B14	DQ099514	I-2-April	Uncultured alpha proteobacterium clone FFW69 (AY828411)	Loch Fyne, UK	*α-Proteobacteria*	98
B15	DQ099515	N-1-April	Uncultured alpha proteobacterium clone CONW51 (AY828396)	Loch Fyne, UK	*α-Proteobacteria*	100
B16	DQ099516	N-1-October	*Mesorhizobium* sp. ICMP 12635 (AY491075)	*Carmichaelia petriei*, New Zeland	*α-Proteobacteria*	98
B17	DQ099517	RB-October	Uncultured bacterium clone RFLP2P (AY962285)	Artesian water supply	*ε-Proteobacteria*	100
B18a	DQ099518	RB-July	Uncultured proteobacterium clone SIMO-855 (AY712392)	Dean Creek Marsh, USA	*α-Proteobacteria*	100
B18b	DQ099519	RB-July	Uncultured bacterium clone Hot Creek 20 (AY168736)	Arsenite-oxidizing biofilm	*δ-Proteobacteria*	90
B19	DQ099520	RB-October	Uncultured bacterium clone PL-29B2 (AY570615)	Oil reservoir, Canada	*ε-Proteobacteria*	100
B20	DQ099521	I-2-January	Uncultured marine bacterium COL-6 (AY028174)	Diatom detritus	γ-*Proteobacteria*	100
B22	DQ099522	RB-July	Uncultured epsilon proteobacterium BioIuz K34 (AF324539)	Unknown	*ε-Proteobacteria*	100
B23	DQ099523	I-2-January	Uncultured gamma proteobacterium clone RAI-3 (AY499429)	*Ria de Aveiro* estuary, Portugal	γ-*Proteobacteria*	98

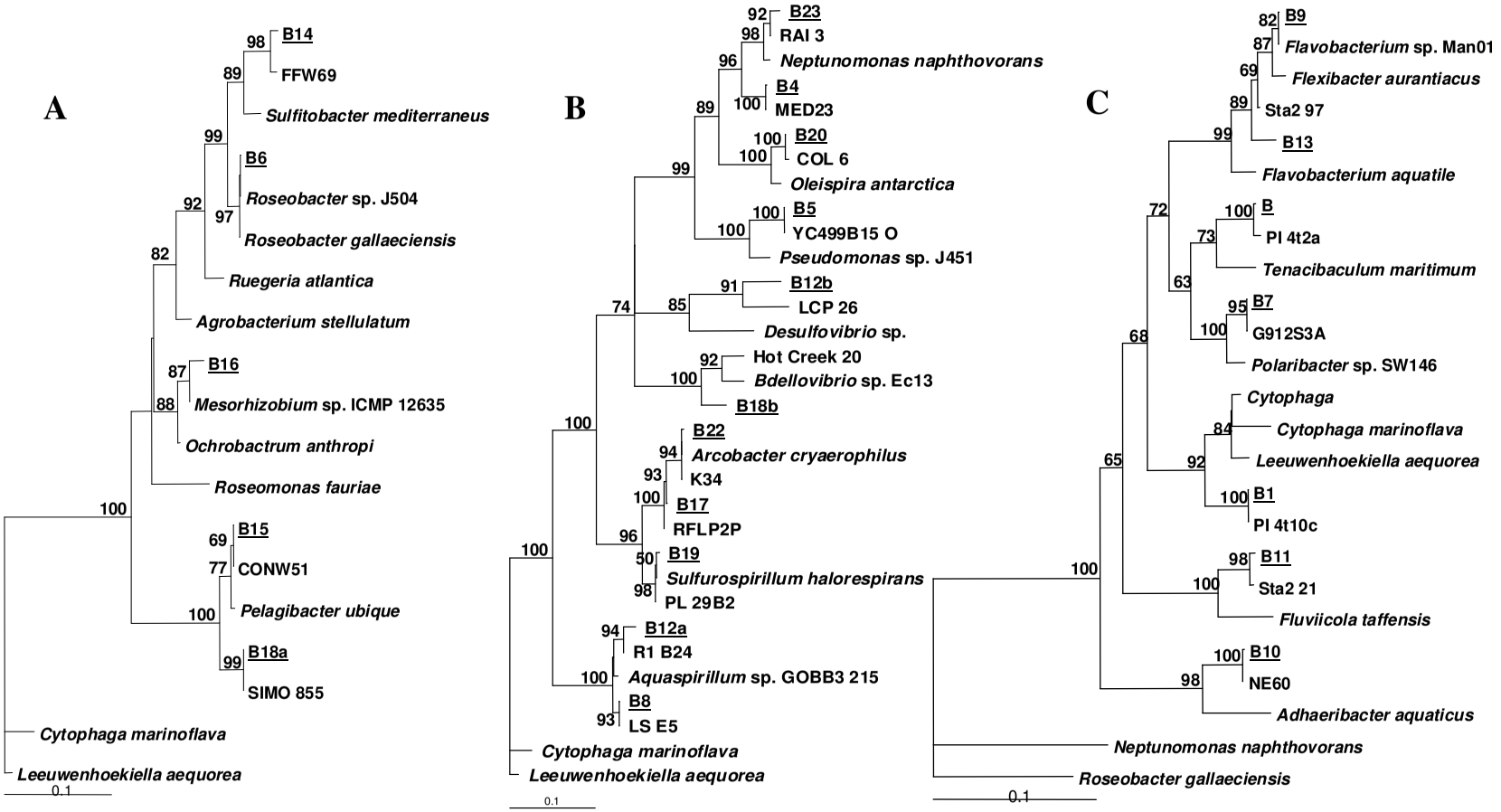

Figure 4.5 Phylogenetic relationships between sequences retrieved from DGGE bands during this study (underlined), reference taxa and available environmental clones, as determined by neighbor-joining analysis (Kimura two-parameter distance optimised criteria) by using approximately 165 nucleotide positions (corresponding to positions 338 to 518 on Escherichia coli 16S rRNA gene): (5A) *α-Proteobacteria* (5B) *β-*, *δ-*, *ε-* and *γ-Proteobacteria* (5C) *Bacteroidetes*. The 16S rRNA gene sequences of *Cytophaga marinoflava* and *Leeuwenhoekiella aequorea* were used as outgroup for the *Proteobacteria* trees, and the 16S rRNA gene sequences of *Roseobacter gallaeciensis* and *Neptunomonas naphthovorans* were used as outgroup for the *Bacteroidetes* tree. Bootstrap support values (1000 replicates) above 50 % are shown adjacent to nodes.

4.4 Discussion

In this study, we applied 16S rDNA PCR-DGGE analysis to characterise the free-living microbial community structure along a salinity gradient in *Ria de Aveiro* and to follow compositional dynamics over several seasons. These features have never been addressed within this estuary.

Cluster and NMDS analysis of DGGE banding patterns revealed that the phylogenetic succession of free-living bacterial community in *Ria de Aveiro* occurred essentially between the brackish and freshwater sections. In fact, major compositional shifts were identified, generally between the brackish community and the communities from sites with salinity lower than 5. These results indicate that bacterial assemblages of distinctly different composition existed in sites with higher and intermediate salinities and in the freshwater section of *Ria de Aveiro*. Similar findings were reported for geographically distinct estuarine systems such as the Parker River estuary (Crump *et al*., 2004), the Rhone River estuary (Troussellier *et al*., 2002) the Tillamook Bay (Bernhard *et al*., 2005), the Moreton Bay (Hewson & Fuhrman, 2004) and the Columbia River estuary (Crump *et al*., 1999).

CCA revealed that salinity and temperature accounted for a significant amount of the variability in the bacterioplankton community composition (26 %). A large fraction of variation (74 %) remained unexplained. As reported for other estuaries, it is probable that factors like residence time, dissolved oxygen and nutrient's composition and concentration are major factors in determining the composition of bacterioplankton community in *Ria de Aveiro* (Bouvier & del Giorgio, 2002; Crump *et al*., 2004). As shown in Table 4.1, a clear longitudinal gradient of salinity was observed between station N-1 and RB. It has been reported that increasing gradients of dissolved oxygen are also established along the salinity gradient and towards the inner estuary (Almeida *et al*., 2002a; Cunha *et al*., 2000; Lopes *et al*., 2005). Nutrient concentrations, namely POC (particulate organic carbon), dissolved PO_4^{3-} and dissolved NO_2^{-} + NO_3^{+} also increase towards the inner estuary, showing pronounced peaks around station I-8 (Almeida *et al*., 2002a; Cunha *et al*., 2000; Lopes *et al*., 2005).

In addition to the effects of the spatial gradient, seasonally driven changes in microbial community in this estuary also occur. Those changes were more evident in the brackish samples from middle estuary, clearly separated in the cluster analysis by

sampling period (Figure 4.2). CCA also highlighted this seasonally driven variability, in some cases probably related to temperature fluctuations (Figure 4.4). For example, bacterial assemblages from station N-1 collected in April, July and October (temperatures ranging from 16 ºC to 17 ºC) are remarkably similar, but clearly distinct from assemblages collected at the same site in January (temperature 14 ºC). On the other hand, communities from station RB collected in April and January are clearly similar, consistent with similar temperature values (15 ºC and 14.5 ºC respectively). Obviously, we are aware that factors such as precipitation levels and organic matter loads, which were not considered in the analysis, and also salinity, are probably implicated in the seasonal related variability. For example, samples collected in July appeared clearly separated from samples collected in other dates (exception made for samples collected from site N1) probably due to higher temperature values but also to lower precipitation levels and higher salinity values registered in this month.

Consistent with the identified spatial shifts, bacterial phylotypes detected in freshwater sites were rarely found at intermediate stations. That may be due to the lower tolerance that freshwater bacteria have to salinity, highlighted by several culture-dependent studies (Hyun *et al*., 1999; Prieur *et al*., 1987). For instance DGGE profiles obtained from site I-10, usually constituted by bands characteristic of the freshwater community, was replaced by a brackish community in summer when salinity in this location raised to 15.7. In this season a clear shift between bacterial assemblages from sites I-10 and RB was not detected (Figure 4.3).

Less pronounced changes were also detected along the salinity gradient in the marine and brackish sections and a few putative brackish specific phylotypes were identified. However, marked differences between brackish and marine communities were not detected and a clearly distinct brackish community in the middle estuary was not identified. This feature was also observed in other estuaries and is usually attributed to short residence times that prevent the development of native bacterial populations (Crump *et al*., 1999, Crump *et al*., 2004). Residence time in *Canal de Ílhavo* ranges from 6 days in spring to several weeks during periods of low freshwater input (Cunha *et al*., 2000). According to these data, a distinct brackish community should be identified in middle estuary at least in summer. However this hypothesis was not confirmed. We suggest that the marine and brackish free-living communities, unlike the freshwater community, are constituted by microorganisms highly tolerant to salinity changes, which

survive within a broad range of salinity conditions. However, differences between those communities may be marked in terms of metabolic activities of their constituents resulting from high metabolic plasticity (Cunha *et al*., 2000; Cunha *et al*., 2001).

Two phylotypes (bands 1 and 6) were detected in all sampling sites with salinity ranging from 0 to 35. Similar findings have been previously reported in studies of estuarine bacterial diversity in San Francisco Bay (Hollibaugh *et al*., 2000), Rhone River estuary (Troussellier *et al*., 2002) and Moreton Bay (Hewson & Fuhrman, 2004).

Analysis of DNA sequences from DGGE bands confirmed that communities from the marine-estuarine and the freshwater sections were phylogenetically distinct and were composed of typical marine and freshwater phylotypes. *Bacteroidetes*, α- and γ-*Proteobacteria* dominated the marine-brackish section. Most of the sequences from this section were 99 to 100 % identical in DNA to sequences from clones or isolates from other marine or estuarine systems like the Plum Island Sound estuary, the Tillamook Bay, the Mediterranean Sea and the Southern ocean (Table 4.2). Those results are in congruence with the study previously described in Chapter 3 regarding the structure of bacterioplankton community within the same sections of this estuary, except that *Bacteroidetes* was not referred then as a dominant group. The study here presented supports the hypothesis that *Bacteroidetes* are often underrepresented in 16S rDNA libraries.

The freshwater section of the estuary was dominated by *Bacteroidetes*, β-, δ- and ε-*Proteobacteria*. Sequences from this section (sites I-10 and RB) were in general closely related to clones or isolates from freshwater systems like Lake Pontchartrain, Lake Grosse Fuchskuhle, Lake Inba, Delaware River or an artesian water supply (Table 4.2).

Studies using different culture-independent approaches reported similar phylogenetic distributions for geographical distinct estuarine systems (Bernhard *et al*., 2005; Castle & Kirchman, 2004; Crump *et al*., 2004; Selje & Simon, 2003). Primarily the dominance of α- and γ-*Proteobacteria* in higher salinity environments and of β-*Proteobacteria* in freshwater environments appears to be common features. Members of the *Bacteroidetes* group are usually found in all marine and freshwater systems frequently as one of the dominant bacterial groups.

Most of the bands exclusive or more pronounced in the middle estuarine section (b5, b20 and b23 in Table 4.2) were affiliated with the γ-*Proteobacteria*. The preference of γ-

Proteobacteria for high nutrient concentration has been reported and peaks of abundance appeared to be related to nutrient point sources (Bouvier & del Giorgio, 2002).

DGGE has recently become a common technique for analysis of bacterial population ecology and dynamics, being particularly useful in studies that involve multiple samples (Muyzer & Smalla, 1998). Notwithstanding its usefulness, data obtained using DGGE should be analysed taking into consideration several associated biases related to DNA extraction efficiency, rRNA gene copy number, PCR primer annealing efficiency, chimera formation, heteroduplex formation and cloning (Farrelly *et al*., 1995; Kopczynski *et al*., 1994; Suzuki & Giovanonni, 1996). Moreover, this technique is able to retrieve only sequences that are present in at least 0.5-1 % of the total cells in the sample (Muyzer *et al*., 1993). Despite these shortcomings, enough evidence has been provided to allow us to assume that this technique presents a valid picture of the community and that the resulting profiles represent the majority of the bacteria present within the bacterial assemblage.

Our results support the hypothesis that estuarine bacterial community distribution is highly related with the salinity gradient. However this relation results not only from the direct effect of salinity variations but also from other factors which co-vary with salinity.

Results here reported suggest that phylogenetic differences between the freshwater and marine-estuarine sections are clearly relevant. However, phylogenetic differences between the estuarine and marine free-living communities are probably not enough to justify different metabolic capabilities previously reported. Those differences are probably related to metabolic adjustments.

5. *Analysing diversity among β-lactamase encoding genes in estuarine waters*

5.1 Introduction

As stated in Chapter 1, during the past decade, concern has grown about the risk that the use and disposal of antibiotics might constitute to human and ecological health (Alonso *et al*., 2001; Kümmerer, 2003). The increasing and often indiscriminate use of antimicrobial drugs in human and veterinary medicine, aquaculture and agriculture has led to water and soil contamination, alteration of microbial ecosystems and, in particular, selection and dissemination of antibiotic-resistant organisms (Alonso *et al*., 2001; Chee-Sanford *et al*., 2001; Schwartz *et al*., 2003). Despite the relevance of this subject, studies concerning antibiotic resistance are usually targeted to bacterial isolates obtained from clinical environments. However, genetic resistance determinants are also undoubtedly present in members of microbial communities from natural environments, many of which are non-cultivable and may hide an unsuspected molecular diversity. The above facts taken together are good reasons for deeper investigations concerning the types of genetic determinants of resistance present in natural environments.

The availability of molecular tools like DNA probes, PCR amplification and DNA sequencing, permit the development of approaches that assess directly the genomic content of each microbial community. Also, the application of exogenous plasmid isolation techniques offers the possibility to characterise not only the molecular diversity of the genetic resistance determinants but also to capture entire functional genes and evaluate the activity of the correspondent enzymes. A DNA-based approach to study the problem of antibiotic resistance avoids the bias introduced by the need of cultivation of resistant bacteria, thus generating a more complete picture of the molecular types of resistant determinants present in a natural environment. This is an important step for risk assessment (Aminov *et al*., 2001; Aminov *et al*., 2002; Chee-Sanford *et al*., 2001; Heuer *et al*., 2002; Schwartz *et al*., 2003).

The resistance to β-lactam antibiotics is a challenging model of study. β-lactams represent the most diverse class of antibiotics, comprising a large variety of structurally different molecules (Demain & Elander, 1999; Kotra & Mobashery, 1998), and including the most commonly prescribed antibacterial agents. It is therefore not surprising that resistance to β–lactam compounds is common and still evolving. Resistance is most frequently related to the production of β–lactamases, which hydrolyse the amide bond of

the β–lactam ring (Kotra & Mobashery, 1998; Bush, 1999). These versatile enzymes are present in both Gram-negative and Gram-positive bacteria and are encoded by genes located on plasmids, transposons or bacterial chromosomes. Based on amino acid sequence composition, β–lactamases are classified into four major molecular classes, A to D (Ambler, 1980). Recently, several β–lactamases displaying novel amino-acid sequences were characterised from environmental isolates: ThinB from *Janthinobacterium lividum* (Rossolini *et al.*, 2001), CAU-1 (Docquier *et al.*, 2002) and Mbl1b (Simm *et al.*, 2001) from *Caulobacter crescentus* and SfhI (Saavedra *et al.*, 2003) and SFC-1 (Henriques *et al.*, 2004) from *Serratia fonticola*.

The extensive use of *Ria de Aveiro* estuarine waters for human activities justifies the need to detect and characterise sequences which could potentially contribute to new antibiotic resistance phenotypes. This work aimed to obtain information about the occurrence of DNA sequences putatively encoding β-lactamases in the estuarine ecosystem and to obtain data on the molecular diversity of those sequences. We also intend to confirm the potential of aquatic environments to represent reservoirs of molecular variants of β–lactamase genes. The study targeted representative groups of clinically relevant β–lactamase genes included in the Ambler classes A, B and D (Ambler, 1980).

The results reinforce the view that environmental gene pools may be important reservoirs of β-lactamase genetic determinants.

5.2 Materials and Methods

5.2.1 Strains and culture techniques

Strains used in this study for testing the primer systems and for generating DNA probes are listed in Table 5.1 and were kindly provided by J.-J. Yan, Department of Pathology, National Cheng Kung University Hospital, Taiwan, China; R. Bonnet, Faculté de Médecine, Service de Bactériologie-Virologie, Université d'Auvergne, Clermont-Ferrand, France; T. M. Coque, Servicio de Microbiologia, Hospital Rámon y Cajal, Madrid, Spain, P. Nordmann, Centre Hospitalier de Bicêtre, Service de Bactériologie-Virologie-Parasitologie-Hygiène, Le Kremlin-Bicêtre, France; A. P. Gibb, Royal Infirmary of

Edinburgh, Medical School, Edinburgh, Scotland, UK.. Strains were grown on Luria-Bertani medium supplemented with ampicillin (50 μg/ml) or imipenem (5 μg/ml) at the adequate temperature with aeration.

Table 5.1 Characteristics of bacterial strains used in this study

Strain	β–lactamase gene	Ambler class	Reference
Klebsiella pneumoniae 6T	TEM-4	A	Coque *et al*., 2002
K. pneumoniae 2s	SHV-2	A	Coque *et al*., 2002
Pseudomonas aeruginosa RP-1	SHV-2a	A	Naas *et al*., 1998
K. pneumoniae Kp40	CTX-M-9	A	Coque *et al*., 2002
Escherichia coli BM21 R^+	CTX-M-10	A	Coque *et al*., 2002
Serratia fonticola UTAD54	FONA derivative	A	Saavedra *et al*., 2003
Pseudomonas putida NTU-92/99	IMP-1	B	Yan *et al*., 2001b
K. pneumoniae KP99c196	IMP-8	B	Yan *et al*., 2001b
P. aeruginosa isolate	IMP-7	B	Gibb *et al*., 2002
P. putida NTU-91/99	VIM-2	B	Yan *et al*., 2001a
P. aeruginosa NTU-39/00	VIM-3	B	Yan *et al*., 2001a
Aeromonas sp.	CphA derivative	B	Tacão *et al*., 2005a
P. aeruginosa isolate	OXA-21	D	De Champs *et al*., 2002
P. aeruginosa SOF1	OXA-31	D	Aubert *et al*., 2001
P. aeruginosa ED-1	OXA-28	D	Poirel *et al*., 2001

5.2.2 Primer design

Ten primer pairs were used to amplify the following genes encoding β–lactamases of three Ambler classes (Ambler, 1980): TEM, SHV, CTX-M and FONA/SFO (Ambler class A); IMP, VIM and Cph-A/IMI-S (Ambler class B) and OXA-1 derivatives, OXA-2 derivatives and OXA-10 derivatives (Ambler class D). The primer sets applied to amplify OXA-like β-lactamase genes were designated OXA-A (designed to amplify genes

codifying β-lactamases included in the OXA-1 group), OXA-B (designed to amplify genes codifying β-lactamases included in the OXA-2 group) and OXA-C (designed to amplify genes codifying β-lactamases included in the OXA-10 group). Primers for SHV, CTX-M, IMP, VIM and OXAB groups were designed during this study. Currently available nucleotide sequences encoding β-lactamases included in the referred groups were downloaded from the GenBank database, which included the following β–lactamase genes (with GenBank accession numbers in parenthesis): SHV: SHV-1 (**AF462396**), SHV-2 (**AY386365**), SHV-5 (**AF462394**), SHV-6 (**Y11069**), SHV-7 (**U20270**), SHV-8 (**U92041**), SHV-11 (**AY293069**), SHV-12 (**AF462395**), SHV-13 (**AF164577**), SHV-14 (**AF226622**), SHV-15 (**AJ011428**), SHV-16 (**AF072684**), SHV-18 (**AF132290**), SHV-24 (**AB023477**), SHV-25 (**AF208796**), SHV-26 (**AF227204**), SHV-27 (**AF293345**), SHV-28 (**AF538324**), SHV-29 (**AF301532**), SHV-34 (**AY036620**), SHV-35 (**AY070258**), SHV-36 (**AF467947**), SHV-37 (**AF467948**), SHV-38 (**AY079099**), SHV-40 (**AF535128**), SHV-41 (**AF535129**), SHV-42 (**AF535130**), SHV-45 (**AF547625**); CTX-M: CTX-M-1 (**AJ416342**), CTX-M-2 (**AJ416343**), CTX-M-3 (**AB059404**), CTX-M-4 (**Y14156**), CTX-M-5 (**AF286192**), CTX-M-9 (**AJ416345**), CTX-M-11 (**AJ310929**), CTX-M-13 (**AF252621**), CTX-M-14 (**AJ416341**), CTX-M-15 (**AF252623**), CTX-M-16 (**AY029068**), CTX-M-17 (**AF454633**), CTX-M-18 (**AF325133**), CTX-M-19 (**AF325134**), CTX-M-20 (**AJ416344**), CTX-M-21 (**AJ416346**), CTX-M-22 (**AY080894**), CTX-M-24 (**AY143430**); IMP: IMP-1 (**AY386702**), IMP-4 (**AF244145**), IMP-5 (**AF290912**), IMP-6 (**AB040994**), IMP-7 (**AF416736**), IMP-8 (**AF322577**), IMP-9 (**AY033653**), IMP-10 (**AB074434**), IMP-11 (**AB074437**); VIM: VIM-1 (**AY152821**), VIM-2 (**AF305559**), VIM-3 (**AF300454**), VIM-4 (**AF531419**), VIM-5 (**AY144612**), VIM-6 (**AY165025**); OXA-2: OXA-34 (**AF350424**), OXA-15 (**U63835**), OXA-32 (**AF315351**), OXA-3 (**L07945**), OXA-2 (**X03037**). Sequences were aligned with the multiple sequence alignment program CLUSTAL X to identify common conserved primer annealing sites (Thompson *et al.*, 1997). The β–lactamases classes A, B and D were treated separately. PCR primers were designed to satisfy specificity. Chosen primer sets were compared to sequences available on the GenBank database to confirm their specificity. The sets of primers used, references, annealing temperatures and predicted amplicon sizes are shown in Table 5.2.

Table 5.2 PCR primers used in this study

Primer pair	Target	Sequence (5′-3′)	Annealing temperature (ºC)	Amplicon size (bp)	Reference
TEM_F	*bla*$_{TEM}$	AAAGATGCTGAAGATCA	44	425	Speldooren, 1998
TEM_R		TTTGGTATGGCTTCATTC			
SHV_F	*bla*$_{SHV}$	GCGAAAGCCAGCTGTCGGGC	62	304	This study
SHV_R		GATTGGCGGCGCTGTTATCGC			
CTX_F	*bla*$_{CTX-M}$	GTGCAGTACCAGTAAAGTTATGG	55	538	This study
CTX_R		CGCAATATCATTGGTGGTGCC			
FONA_F	*bla*$_{FONA}$	GATCGATACCGCCGATAATTCGC	60	550	Saavedra *et al*., 2003
FONA_R		ACGGCGATATCGTTAGTGGTACC			
IMPF	*bla*$_{IMP}$	GAATAGAGTGGATTAATTCTC	55	232	This study
IMPR		GGTTTAAYAAAACAACCACC			
VIMF	*bla*$_{VIM}$	GATGGTGTTTGGTCGCATATCG	58	475	This study
VIMR		GCCACGTTCCCCGCAGACG			
AER_F	*bla*$_{CphA/IMIS}$	GCCTTGATCAGCGCTTCGTAGTG	60	670	This study
AER_R		GCGGGGATGTCGCTGACGCAG			
OXAA_F	*bla*$_{OXA-1}$ derivatives	ACACAATACATATCAACTTCGC	53	814	Ouellette *et al*., 1997
OXAA_R		AGTGTGTTTAGAATGGTGATC			
OXAB_F	*bla*$_{OXA-2}$ derivatives	CAAGCCAAAGGCACGATAGTTG	56	561	This study
OXAB_R		CTCAACCCATCCTACCCACC			
OXAC_F	*bla*$_{OXA-10}$ derivatives	CGTGCTTTGTAAAAGTAGCAG	53	652	Huovinen *et al*., 1988
OXAC_R		CATGATTTTGGTGGGAATGG			

5.2.3 Site description and sample collection

For this study, water samples were obtained from three sampling sites within a section of the estuary (*Canal de Ilhavo*) (see Figure 2.1 – **Chapter 2**): station N-1 close to the mouth of the lagoon, representing the deep marine zone subjected to anthropogenic pressure mainly by the presence of harbour facilities, and stations I-6 and I-10, representing the shallow inner brackish water zone where the main sources of contamination are aquaculture ponds, industrial plants, diffuse domestic sewage inputs and run-off from agriculture fields. Samples were collected in November 2003, always during daytime, at low tide, approximately 0.2 m below the surface, by immersion of 2 l autoclaved bottles.

5.2.4 DNA extraction

Total genomic DNA was isolated from pure cultures of bacterial strains by using the Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania), according to the manufacturer's instructions. From water samples, DNA was extracted in the laboratory within 1 h of sampling. Depending on the sample bacterial density, 450 ml (for station I-10), 1 l (for station I-6) and 3 l (for station N-1) of water were pre-filtered through 5-µm-pore-size polycarbonate filters (Poretics Products, Livermore, USA) and then bacterial cells were collected on 0.2-µm-pore-size filters (Poretics Products, Livermore, USA). Filters were immediately washed with 2 ml of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and suspended particles were harvested by centrifugation (15 200 g, 10 min.). DNA extraction was performed using two commercial systems: with the UltraClean™ Soil DNA isolation Kit (MoBio Inc., Solana, CA, USA), DNA extraction was performed as described by the manufacturer, using 0.25 mg of the harvested pellet; when using the Genomic DNA Extraction Kit (MBI Fermentas, Vilnius, Lithuania), the pellet (approximately 0.125 mg) was resuspended in 200 µl TE buffer containing 10 mg/ml lysozyme, incubated for 1 h at 37 ºC and then frozen and thawed three times, followed by extraction according to the manufacturer's instructions. DNA was aliquoted in TE buffer and stored at -20 ºC.

5.2.5 PCR amplification

PCR reactions were performed using as substrate genomic DNA or heat–denatured cells from pure cultures of the positive controls. Also, equal amounts of total DNA extracted from environmental samples with the two methods referred were used in the PCR experiments. 50 µl reaction mixtures contained 1X PCR buffer (PCR buffer without $MgCl_2$:PCR buffer with $(NH_4)_2SO_4$, 1:1), 3 mM $MgCl_2$, 5 % dimethylsulfoxide, 200 µM each nucleotide, 15 pmol of each primer, 1 U of *Taq* polymerase, and 50-100 ng of purified DNA (5 µl of heat-denatured cells). The temperature profile was as follows: initial denaturation (94 ºC for 9 min); 30 cycles of denaturation (94 ºC for 30 s), annealing (as indicated in Table 5.2 for 30 s), and extension (72 ºC for 1 min 30 s); and a final extension (72 ºC for 10 min). All PCRs were carried out with *Taq* polymerase, nucleotides and buffers purchased from MBI Fermentas (Vilnius, Lithuania) in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Richmond, CA, USA). PCR products (5 µl) were analysed by electrophoresis on a 2 % agarose gel and stained with ethidium bromide. To define the detection limit of the primer sets, genomic DNA from the positive control strains was used. Serial dilutions were prepared starting from solutions of genomic DNA at 100 µg/ml. The DNA concentrations in the PCR solutions ranged from 100 ng and 1 fg (in 10 fold dilutions).

5.2.6 Southern blot hybridisation

Hybridisation studies with PCR products obtained using each primer set were performed to confirm specificity. Intragenic digoxigenin-labelled probes were obtained from positive controls by PCR, using primers and PCR conditions (see Table 5.2) as described above except that PCR Dig Labelling Mix (Roche Molecular Biochemicals, Indianapolis, USA) was included in the reaction mixture instead of dNTPs. PCR products were separated on 2 % agarose gels, vacuum transferred to nylon membranes (Roche Molecular Biochemicals, Indianapolis, USA) and crosslinked by UV irradiation for 5 min. Membranes were prehybridised for 2 h at 42 ºC in hybridisation buffer (50 % formamide, 5x SSC, 0.1 % N-lauroylsarcosine, 0.02 % SDS, 1 % Blocking Reagent), and hybridised for

12 to 16 h at 42 ºC in the same buffer. Two 5 min washes in 2x SSC, 0.1 % SDS at room temperature were followed by other two 15 min washes at 42 ºC, in 0.5x SSC, 0.1 % SDS. Detection was performed using the DIG Nucleic Acid Detection Kit following instructions provided by the manufacturer (Roche Molecular Biochemicals, Indianapolis, USA). Positive and negative controls were included in all experiments to confirm the specificity of detection.

5.2.7 Cloning of PCR amplicons and DGGE analysis

DNA sequences corresponding to TEM, IMP and OXA-B β-lactamase genes were amplified from station I-10 as described above except that the proof reading *Pfu* DNA polymerase (MBI Fermentas, Vilnius, Lithuania) was used to minimise the chance of errors introduced during amplification. PCR products were cloned into cloning vector pCR®-Blunt (Kan^R, Zeo^R) using the Zero Blunt® PCR Cloning Kit (Invitrogen, Carlsbad, California, USA) and transformed into *Escherichia coli* TOP10 competent cells according to the manufacturer's instructions. Kanamycin-resistant colonies were randomly picked and screened by PCR for the presence of β-lactamase fragments by using the same primer sets that were used for amplification, except that the forward primer included a GC clamp at the 5' end. DNA of each clone was obtained by picking the colony with a sterile toothpick, suspending the cells in 5 µl sterile water and incubating for 10 min at 100 ºC. The lysate obtained was added to the PCR mix. The PCR conditions were as described above.

DGGE was performed with a DCode™ Universal Mutation Detection System (Bio-Rad, Richmond, CA, USA). The PCR products were loaded onto a polyacrylamide gel (8 % [wt/vol] acrylamide in 0.5X TAE buffer) with 20-30 % to 40-50 % denaturant gradients (100 % denaturant gradient was 7M urea and 40 % deionised formamide). Electrophoresis was carried out in 0.5X TAE buffer at 20 V for 15 min followed by 5 h and 30 min at 200 V, and a temperature of 60 ºC. After electrophoresis, the gels were stained for 5 min with ethidium bromide (0.5 µg/l solution) and then washed in distilled water for 20 min. Gel images were acquired using a Molecular Imager FX™ system (Bio-Rad, Richmond, CA, USA) and analysed with the Diversity Database™ Fingerprinting software (Bio-Rad, Richmond, CA, USA).

5.2.8 Sequencing and phylogenetic analysis

PCR products were purified with the Concert™ Rapid PCR Purification System (Gibco BRL, Eggenstein, Germany) and used as template in the sequencing reactions, carried out with the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, California, USA). The reaction mixtures were analysed in an automatic DNA sequencer (ABI PRISM® 310 Genetic Analyser, PE Applied Biosystems). Sequences were edited manually after examination of the corresponding chromatogram files. On line similarity searches were performed using the BLAST program (Altschul *et al.*, 1997). Sequences were translated using the Translate Tool available at the Swiss Institute of Bioinformatics Expasy website (**http://www.expasy.org/tools/dna.html**).

The nucleotide sequences determined in this study and β–lactamase gene sequences previously reported and available in GenBank databases were aligned using the Clustal X program (Thompson *et al.*, 1997). Phylogenetic analyses were performed with PAUP* version 4.0b10 (Swofford, 2003). Three trees corresponding to Ambler classes A, B (subclass B1) and D were produced using the neighbour-joining tree-building algorithm. To root the trees, the following gene sequences were used as outgroups: $bla_{\text{OXA-1}}$ for class A tree, $bla_{\text{TEM-1}}$ for class D and bla_{CphA}, $bla_{\text{Sfh-I}}$ and bla_{ImiS} for class B1. Bootstrap values (1000 replicates) were determined.

Nucleotide sequence accession numbers: Nucleotide sequences retrieved that shared less than 97 % similarity with genes previously reported were deposited in the GenBank nucleotide database under the following accession numbers: **AY663805** (IM9), **AY663806** (IM7), **AY663807** (O3) and **AY663808** (O5).

5.3 Results

5.3.1 Design and validation of PCR primers targeting β–lactamase encoding sequences

DNA sequences encoding β-lactamases were aligned in order to design primers targeting conserved regions of the genes. For each set of primers, annealing temperatures were optimised experimentally in order to obtain specific amplification. PCR conditions were rigorously tested using DNA and colony biomasses of control strains as substrate: in all cases the observed size of the amplicons corresponded to the expected size (Figure 5.1). Strains containing other β–lactamase genes were used as negative controls and no signal was produced in incongruent primer-template combinations. Negative controls consisting of PCR reactions with the same composition except that water was added instead of DNA were also used. The nucleotide composition of the amplicons obtained from control strains was determined by DNA sequencing. In all cases the sequence was identical to the expected, thus confirming the specificity of the primers and the fidelity of the amplification.

Probes specific for each of the β–lactamase genes under analysis were generated by incorporation of digoxigenin during PCR amplifications using the adequate primer pair and DNA from the correspondent positive control strain. Specificities of the probes were confirmed against the fragments amplified from control strains (Figure 5.1).

5.3.2 PCR amplification and hybridisation of bla sequences from estuarine water

DNA from bacterial assemblages was obtained from three sites in the *Ria de Aveiro* lagoon: N-1, I-6 and I-10. DNA was extracted using two different commercial systems in a comparative manner: UltraClean™ Soil DNA isolation Kit (MoBio Inc., Solana, CA, USA) and Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania). The primer pairs presented in Table 5.2 were used to drive amplification of *bla* sequences from environmental DNA. After gel electrophoresis, the nature of the resulting bands was

confirmed by hybridisation (Figure 5.1). Although primer sets performed well with pure cultures, IMP and TEM primers gave weak non-specific products when used with total community DNA (Figure 5.1A and 5.1I). The non-specific nature of these bands was confirmed by hybridisation (Figure 5.1B and 5.1J).

As summarized in Table 5.3, with the exception of CTX-M-like genes, DNA sequences representing all groups of β–lactamase genes were detected in water samples from *Ria de Aveiro*, revealing a high prevalence and diversity of these resistance genes in this environment. Amplicons representing $bla_{\text{OXA-A}}$ and $bla_{\text{OXA-C}}$ genes were not detected in N-1 station (Figure 5.1M and 5.1Q) and in the same sample an amplicon representing SHV sequences was only detected after hybridisation with a specific probe (Figure 5.1C and 5.1D). Additionally and taking into consideration band intensity, most of the *bla* representing sequences seem to be more abundant in the I-10 site and less abundant in site N-1 (Figure 5.1). Contrasting results were obtained with fragments representing the VIM gene: amplicons obtained from N-1 DNA were directly visible but from the I-10 sample were only detected after hybridisation and were not detected in I-6 DNA (Figure 5.1; Table 5.3). Also the SHV gene was not detected in samples collected from the I-10 site (Figure 5.1; Table 5.3).

Table 5.3 β–lactamase genes detected in total DNA from estuarine waters

Sample	β–lactamase genes									
	TEM	**SHV**	**CTX-M**	**FONA**	**IMP**	**VIM**	**CphA/IMI-S**	**OXA-A**	**OXA-B**	**OXA-C**
N-1	+	+[a]	-	+	+	+	+	+	-	-
I-6	+	+	-	+	+	-	+	+	+	+
I-10	+	-	-	+	+	+[a]	+	+	+	+

[a] amplicon detected only by hybridisation

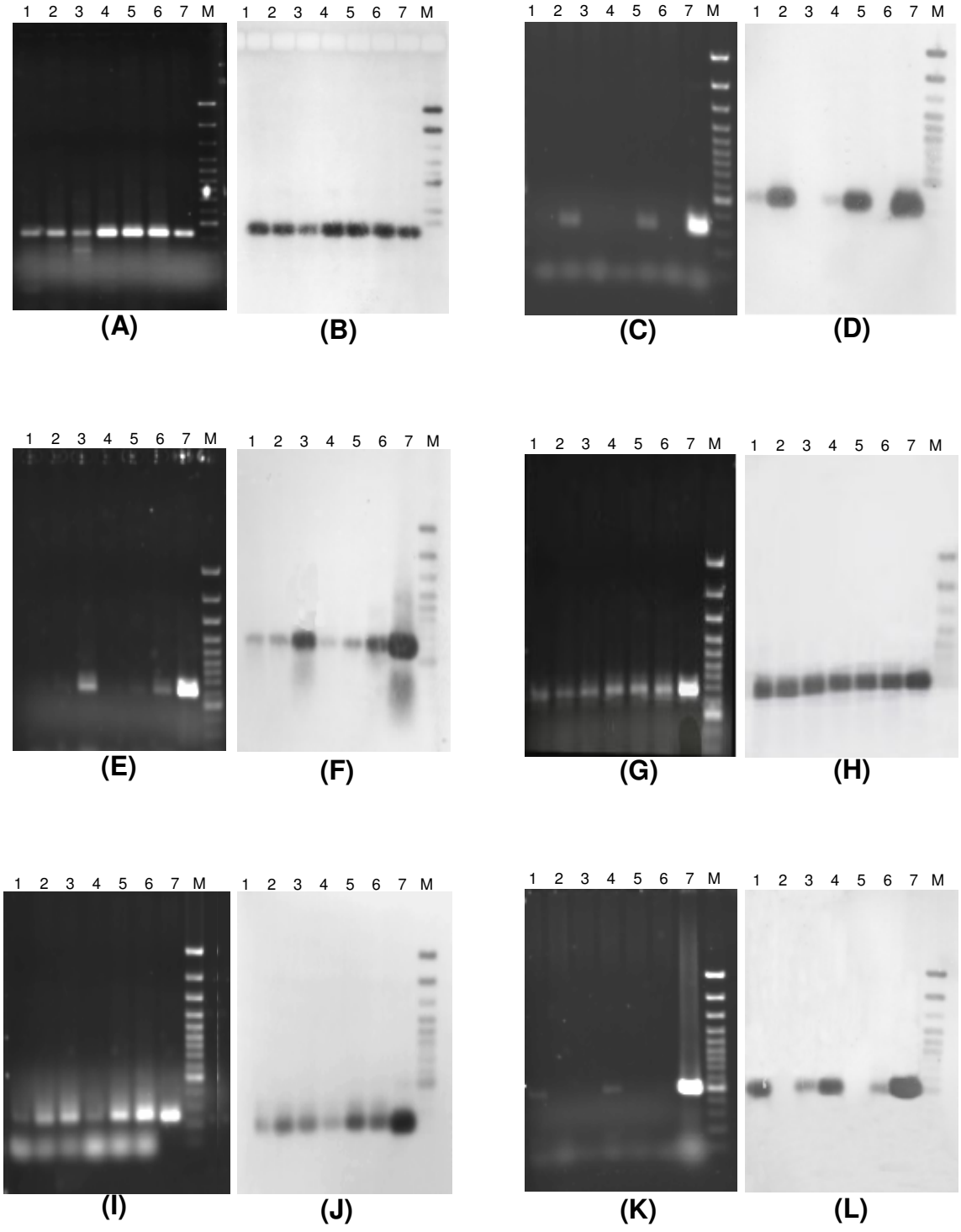

Figure 5.1 See next page

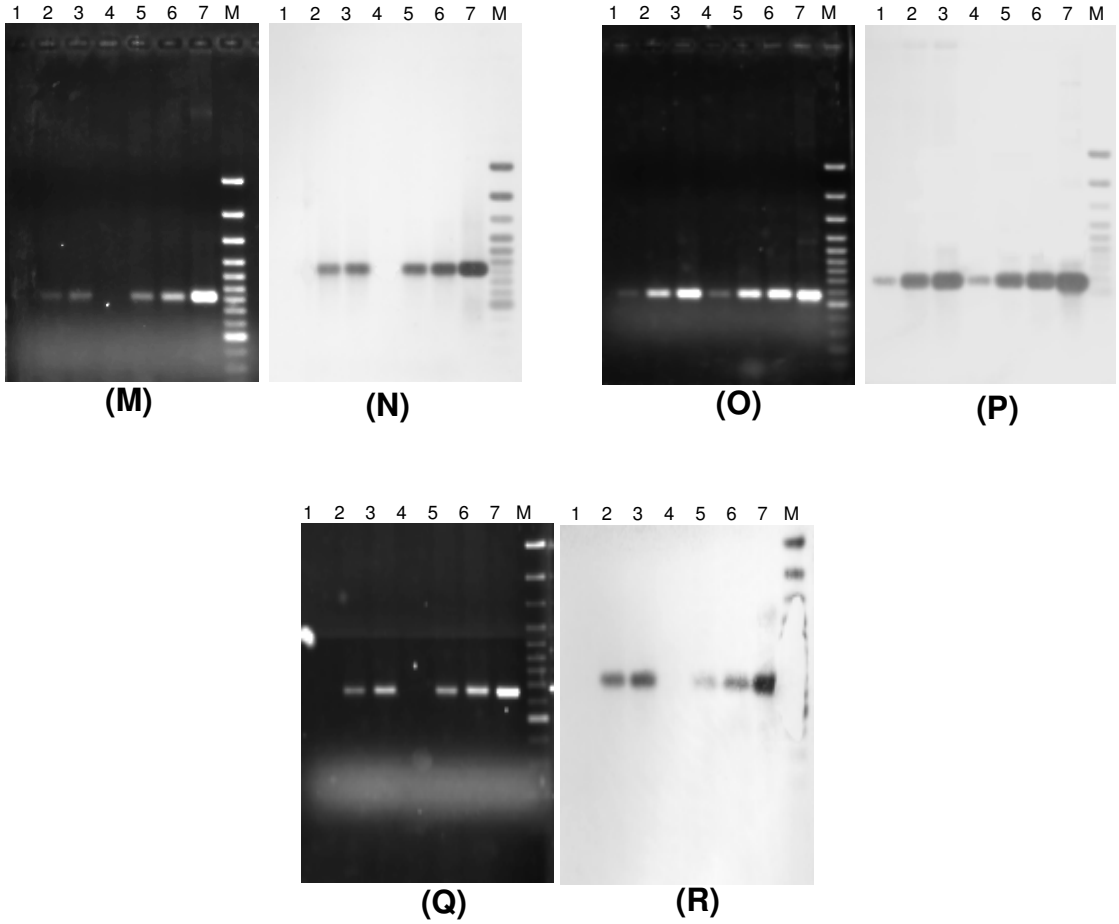

Figure 5.1 Detection of β-lactamase genes related to bla_{TEM} (A, B), bla_{SHV} (C, D), bla_{FONA} (E, F), bla_{CphA} (G, H), bla_{IMP} (I, J), bla_{VIM} (K, L), $bla_{OXA\text{-}A}$ (M, N), $bla_{OXA\text{-}B}$ (O, P) and $bla_{OXA\text{-}C}$ (Q, R) in water samples from sites N-1 (lanes 1 and 4), I-6 (lanes 2 and 5) and I-10 (lanes 3 and 6) in the estuarine system *Ria de Aveiro*. Detection was achieved by PCR amplification (A, C, E, G, I, K, M, O and Q) and hybridisation of Southern-blotted PCR products (B, D, F, H, J, L, N, P and R). Genomic DNA was extracted using the commercial systems Soil DNA Isolation Kit (lanes 1, 2 and 3) and Genomic DNA Purification Kit (lanes 4, 5 and 6). A positive control was included (lane 7). Lane 8: DNA size marker GeneRuler™ 100 bp DNA ladder plus (MBI Fermentas, Vilnius, Lithuania).

5.3.3 DGGE and sequence analysis

Each amplicon obtained from environmental DNA may include sequences of diverse nucleotide composition. Molecular diversity among amplicons representing TEM, IMP and OXA-B groups of β-lactamases (representatives of the Ambler classes A, B and D, respectively) was inspected. The lower limits of PCR detection of primers used to amplify these genes were calculated: the limits of TEM and IMP primers ranged from 1 pg and 10 pg and of OXA-B primers ranged from 10 fg and 100 fg. Three libraries were constructed by direct cloning of PCR products from sampling site I-10. This sampling site was chosen due to the fact that, considering band intensity, β–lactamase gene sequences (particularly sequences representing bla_{IMP} *and* $bla_{OXA\text{-}B}$) seem to be more common at site I-10 (Figure 5.1). Twenty-four clones from each library were selected and the behaviour of the inserts in DGGE gels was analysed (Figure 5.2). Clones containing inserts displaying different DGGE types were further characterised by DNA sequencing. The β–lactamase fragments amplified from positive controls were also sequenced to determine polymerase chain reaction fidelity: we did not detect errors introduced by the polymerisation reaction.

Diversity among TEM representing sequences: A 30-50 % denaturant gradient was used to separate TEM fragments (Figure 5.2A). Three band positions (designated T1, T2 and T3) were identified. T1 was the most frequent DGGE type (21 out of 24 clones) followed by T2 (2 clones out of 24) and T3 (1 clone out of 24). Sequence analysis revealed that the three sequence types shared a high percent similarity (>99 %). DNA sequences from representatives of the T1 band position were 100 % identical to the corresponding region in TEM-116 gene (GenBank accession number **AAQ95605**). For band type T2, 3 nucleotide substitutions were found, resulting in only one amino-acid substitution from Leucine to Serine at position 40 according to Ambler numbering scheme (Ambler *et al.*, 1991) (as far as we know, never described in previous studies) (Figure 5.3). The DNA sequence of band type T3 is characterised by one silent nucleotide substitution.

Diversity among IMP representing sequences: A 20-40 % denaturant gradient was used to separate IMP fragments (Figure 5.2B). Nine band positions were identified designated IM1 to IM9. IM6 was the most frequent DGGE type including 6 clones out of 24. DNA sequences shared a high percent similarity between them, and the majority showed high percent similarity to the previously characterised IMP-5 gene (da Silva *et al.*,

2002). However, only IM1 sequence (3 clones) was 100 % identical to bla_{IMP-5}. In most of the retrieved sequences (or deduced amino-acid sequences) silent mutations or amino acids substitutions previously reported were identified. Substitutions never reported before were detected in two band types: for IM6, an amino acid substitution from Aspartate to Glycine at position 150, and for IM8, an amino acid substitution from Glycine to Aspartate at position 151, according to the numbering of Bc-II from *Bacillus cereus* 569/H (Galleni *et al.*, 2001) (Figure 5.3). DNA sequences retrieved from clones with patterns IM7 and IM9 were most closely related to the IMP-2 gene (Riccio *et al.*, 2000). However, the IM7 sequence shared only 94 % nucleotide identity with this gene and IM9 shared only 90 % sequence identity. The deduced amino-acid sequence of IM9 showed two amino acid substitutions that have never been reported (Leucine to Isoleucine at position 146 and Valine to Alanine at position 169) (Figure 5.3).

Diversity among OXA-B representing sequences: A 20-50 % denaturant gradient was used to separate OXA-2-relatives fragments (Figure 5.2C). Five band positions were identified in DGGE gels named O1 to O5. O1 was the most frequent DGGE type including 16 clones out of 24. The nucleotide sequence retrieved from O1 clones was 100 % identical to OXA-2 (Dale *et al.*, 2005; Nucken *et al.*, 1989). Nucleotide sequences retrieved from O2 (1 clone) and O4 (1 clone) fragments also shared a high percent similarity with OXA-2 gene (>97 %), possessing silent mutations (O2) or resulting in amino acids substitutions previously reported (O4) (Sanschagrin *et al.*, 1995; Vila *et al.*, 1997) (Figure 5.3). The O3 nucleotide sequence (5 clones) and the O5 nucleotide sequence (1 clone) shared only 84 % similarity to the corresponding region of the OXA-21 gene (de Champs *et al.*, 2002) and the OXA-20 gene (Naas *et al.*, 1998), respectively.

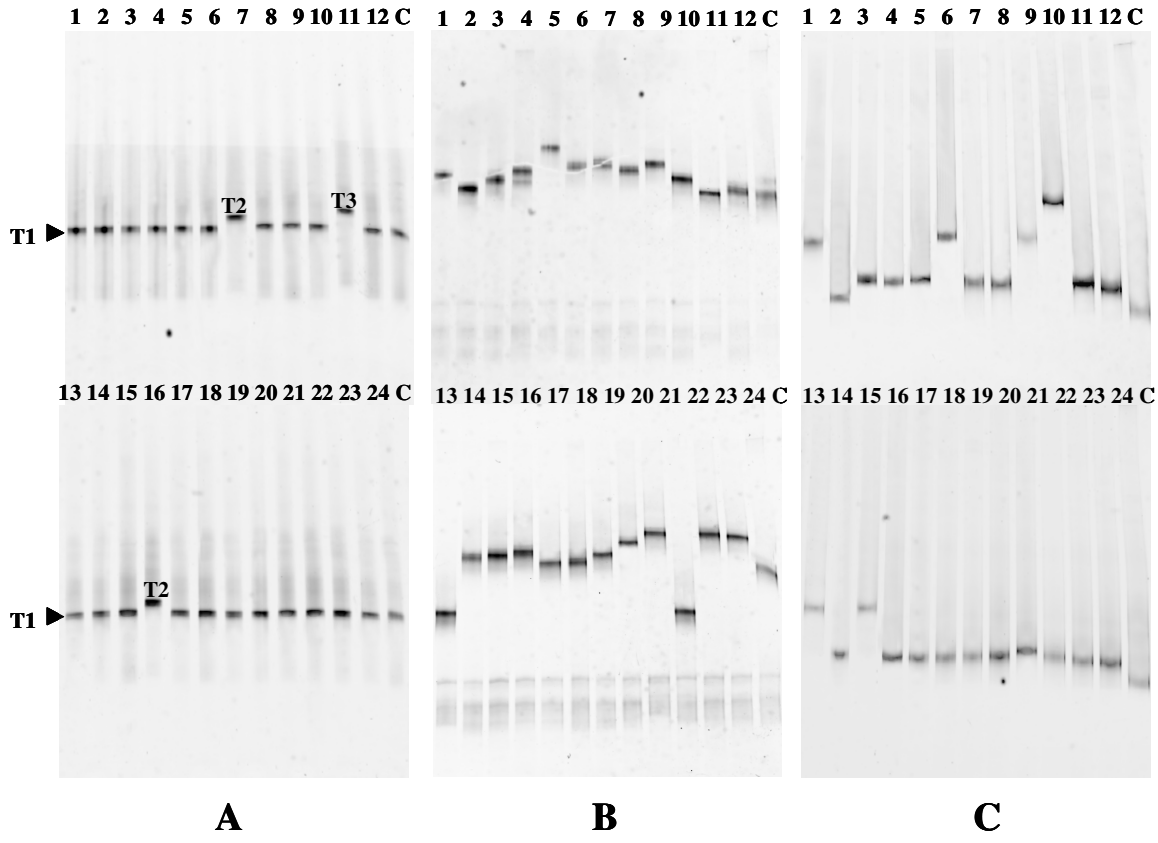

Figure 5.2 DGGE analysis of β-lactamase gene fragments amplified from clones (lanes 1-24) picked from TEM (A), IMP (B) and OXA-B (C) genomic libraries. Lanes C, fragment amplified from positive controls of the corresponding β-lactamases. In Figure 5.2A the band positions T1, T2 and T3 are indicated. For Figure 5.2B the correspondences between band positions and clones are: band position IM1 - clones 1, 3, 10; IM2 - 4, 6, 8; IM3 – 5; IM4 – 7, 20; IM5 – 9; IM6 - 2, 12, 14, 15, 16, 19; IM7 – 13, 22; IM8 - 21, 23, 24 and IM9 - 11, 17, 18; for Figure 5.2C the correspondences are band position O1- clones 2, 4, 5, 6, 7, 8, 10, 11, 12, 15, 16, 17, 19, 20, 23, 24; O2 – 9; O3 - 1, 3, 13, 18, 21; O4 - 14 and O5 - 22.

(A)

```
                                                                  50
TEM-1           MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRP 60
TEM-116         MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRP 60
T3              -------------------------------KDAEDQLGARVGYIELDLNSGKILESFRP 29
T1              -------------------------------KDAEDQLGARVGYIELDLNSGKILESFRP 29
T2              -------------------------------KDAEDQSGARVGYIELDLNSGKILESFRP 29

                       70                  90                  110
TEM-1           EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL 120
TEM-116         EERFPMMSTFKVLLCGAVLSRIDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL 120
T3              EERFPMMSTFKVLLCGAVLSRIDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL 89
T1              EERFPMMSTFKVLLCGAVLSRIDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL 89
T2              EERFPMMSTFKVLLCGAVLSRIDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL 89

                     130                 150
TEM-1           CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM 180
TEM-116         CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM 180
T3              CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPE-------------- 135
T1              CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPE-------------- 135
T2              CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPE-------------- 135
```

(B)

```
                                                              130
IM1             ---------------------------------------------IEWLNSQSIPTYASELT 17
IMP-5           EAYLIDTPFTAKDTEKLVTWFVERGYKIKGSISSHFHSDSTGGIEWLNSQSIPTYASELT 120
IM4             ---------------------------------------------IEWLNSQSIPTYASELT 17
IM8             ---------------------------------------------IEWLNSQSIPTYASELT 17
IM3             ---------------------------------------------IEWLNSQSIPTYASELT 17
IM6             ---------------------------------------------IEWLNSQSIPTYASELT 17
IM5             ---------------------------------------------IEWLNSQSIPTYASELT 17
IM2             ---------------------------------------------IEWLNSQSIPTYASELT 17
IM9             ---------------------------------------------IEWLNSQSIPTYASELT 17
IMP-2           DAYLIDTPFTATDTEKLVNWFVERGYKIKGTISSHFHSDSTGGIEWLNSQSIPTYASELT 120
IM7             ---------------------------------------------IEWLNSQSIPTYASELT 17

                     150             180                 200
IM1             NELLKKDGKVQAKNSFSGASYWLVKKKIEVFYPGPGHTPDNVVVWLPENR---------- 67
IMP-5           NELLKKDGKVQAKNSFSGASYWLVKKKIEVFYPGPGHTPDNVVVWLPENRVLFGGCFVKP 180
IM4             NELLKKDGKVQAKNSFSGASYWLVKKKIEVFYPGPGHTPDNVVVWLPENR---------- 67
IM8             NELLKKDDKVQAKNSFSGVSYWLVKKKIEVFYPGPGHTPDNVVVWLPENR---------- 67
IM3             NELLKKDGKVQAKNSFSGVSYWLVKKKIEVFYPGPGHTPDNVVVWLPENR---------- 67
IM6             NELLKKGGKVQAKNSFSGVSYWLVKKKIEVFYPGPGHTPDNVVVWLPENR---------- 67
IM5             NELLKKDGKVQAKNSFSGVSYWLVKNKIEVFYPGPGHTPDNVVVWLPEN----------- 66
IM2             NELLKKDGKVQAKNSFSGVSYWLVKNKIEVFYPGPGHTPDNVVVWLPE------------ 65
IM9             NEILKKDGKAQAKNSFSGVSYWLVKNKIEVFYPGPGHTPDNVVVWLPEKK---------- 67
IMP-2           NELLKKDGKVQAKNSFSGVSYWLVKNKIEVFYPGPGHTQDNVVVWLPEKKILFGGCFVKP 180
IM7             NELLKKNGKVQAKNSFSGVSYWLVKNKIEVFYPGPGHTPDNVVVWLPEKK---------- 67
```

Figure 5.3 See next page.

(C)

```
                                                                50
O1              ------------------------------------------------ADERQADRAMLVFD 14
O2              ------------------------------------------------ADERQADRAMLVFD 14
OXA-2           MAIRIFAILFSIFSLATFAHAQEGTLERSDWRKFFSEFQAKGTIVVADERQADRAMLVFD 60
O4              ------------------------------------------------ADERQADRAMLVFD 14
OXA-21          MAIRIFAILFSTFVFGTFAHAQEGMRERSDWRKFFSEFQAKGTIVVADERQTDRVILVFD 60
O3              ------------------------------------------------ADERQADRILSVFD 14
O5              ------------------------------------------------ADERANVRSTSVYD 14
OXA-20          MIIRFLALLFSAVVLVSLGHAQEKTHESSNWGKYFSDFNAKGTIVVVDERTNGNSTSVYN 60

                      70                      90                      110
O1              PVRSKKRYSPASTFKIPHTLFALDAGAVRDEFQIFRWDGVNRGFAGHNQDQDLRSAMRNS 74
O2              PVRSKKRYSPASTFKIPHTLFALDAGAVRDEFQIFRWDGVNRGFAGHNQDQDLRSAMRNS 74
OXA-2           PVRSKKRYSPASTFKIPHTLFALDAGAVRDEFQIFRWDGVNRGFAGHNQDQDLRSAMRNS 120
O4              PVRSKKRYSPASTFKIPHTLFALDAGAVRDEFQIFRWDGVNRGFAGHNQDQDLRSAMRNS 74
OXA-21          QVRSEKRYSPASTFKIPHTLFALDAGAARDEFQVFRWDGIKRSFAAHNQDQDLRSAMRNS 120
O3              QARATKRYSPASTFKIPHTLFALDVGAVRDEFQVFRWDGIKRSFAGHNQDQNLRSAMRNS 74
O5              EVRAQQRYSPASTFKIPHTLFALDAGAVRDEFQIFQWDGVKRGFAGHNQNQDLRSAMRTS 74
OXA-20          ESRAQQRYSPASTFKIPHTLFALDAGAVRDEFHVFRWDGAKRSFAGHNQDQNLRSAMRNS 120

                        130                     150             170
O1              TVWVYELFAKEIGDDKARRYLKKIDYGNADPSTSNGDYWIEGSLAISAQEQIAFLRKLYR 134
O2              TVWVYELFAKEIGDDKARRYLKKIDYGNADPSTSNGDYWIEGSLAISAQEQIAFLRKLYR 134
OXA-2           TVWVYELFAKEIGDDKARRYLKKIDYGNADPSTSNGDYWIEGSLAISAQEQIAFLRKLYR 180
O4              TVWVYELFAKEIGDDKARRYLKQIDYGNADPSTSNGDYWIDGNLAIAAQEQIAFLRKLYH 134
OXA-21          TVWIYELFAKEIGEDKARRYLKQIDYGNADPSTSNGDYWIDGNLAIAAQEQIAFLRKLYH 180
O3              TVWVYELFAREIGEEKAKRYLKQIGYGNAGPSTSKGDYWIDGTLEISAYEQISFLRKLYR 134
O5              AIWVYQLFAKEIGEDNAQSYLKKINYGNADPTTKSGDYWVDGNLAISAHEQISFLKSLYR 134
OXA-20          TVWVYQLFAKEIGENKARSYLEKLNYGNADPSTKSGDYWIDGNLAISANEQISILKKLYR 180

                    190             200
O1              NELPFRVEHQRLVKDLMIVEAGRNWILRAKTGWEGRMG---------------------- 172
O2              NELPFRVEHQRLVKDLMIVEAGRNWILRAKTGWEGRMG---------------------- 172
OXA-2           NELPFRVEHQRLVKDLMIVEAGRNWILRAKTGWEGRMGWWVGWVEWPTGSVFFALNIDTP 240
O4              NELPFRVEHQRLVKDLMIVEAGRNWILRAKTGWEGRMG---------------------- 172
OXA-21          NELPFRVEHQRLVKDLMIVEAGRNWILRAKTGWEGRMGWWVGWVEWPTGPVFFALNIDTP 240
O3              NELPFRVEHQRLVKDLMITEAGRNWILRAKTGWK-------------------------- 168
O5              NELPFRVEHQRLVKDIMIVEARRDWILRAKTGW--------------------------- 167
OXA-20          NELPFRVEHQRLVKDLMIVEAKRDWILRAKTGWDGQMGWWVGWVEWPTGPVFFALNIDTP 240
```

Figure 5.3 Alignments of deduced amino-acid sequences retrieved from TEM (alignment A), IMP (alignment B) and OXA-2 (alignment C) libraries with most closely related β-lactamase sequences downloaded from the GenBank database. Numbering for TEM β-lactamases is according to Ambler numbering scheme (Ambler, 1991), for IMP β-lactamases is according to the numbering of Bc-II from *Bacillus cereus* 569/H (Galleni *et al*., 2001) and for OXA β-lactamases is according to the class D β-lactamase numbering (Couture *et al*., 1992).

5.3.4 Phylogenetic analysis

The phylogeny of the sequences retrieved in this study was investigated. For that, three phylogenetic trees were built using nucleotide sequences from previously characterised β–lactamase genes and the sequences obtained in this study (Figure 5.4).

Figure 5.4A presents a phylogenetic tree for Ambler class A β–lactamases where several previously reported clusters can be identified. The TEM clade includes the sequences retrieved during this study (T1, T2 and T3). Sequences T1, T2 and T3 belong to a sub-cluster that, according to this analysis, appear to be more ancient than the other TEM sequences included.

Figure 5.4B presents the phylogenetic placement of several subclass B2 β-lactamases previously characterised and the sequences retrieved during this study (IM1 to IM9). As stated before, IM sequences are closely related to the IMP sequences previously characterised and the phylogenetic analysis shows that they are widely distributed within the IMP clade.

Figure 5.4C presents a phylogenetic analysis of the class D β-lactamases. Sequences downloaded from the databases and sequences retrieved from the environmental libraries (O1 to O5) were included. Analysis of the resulting tree reveals that environmental sequences group with the OXA-2 related sequences, and that they are widely distributed within this clade. Some of the retrieved sequences, namely O3 and O5, appear at the root of this clade, revealing a probably ancient origin.

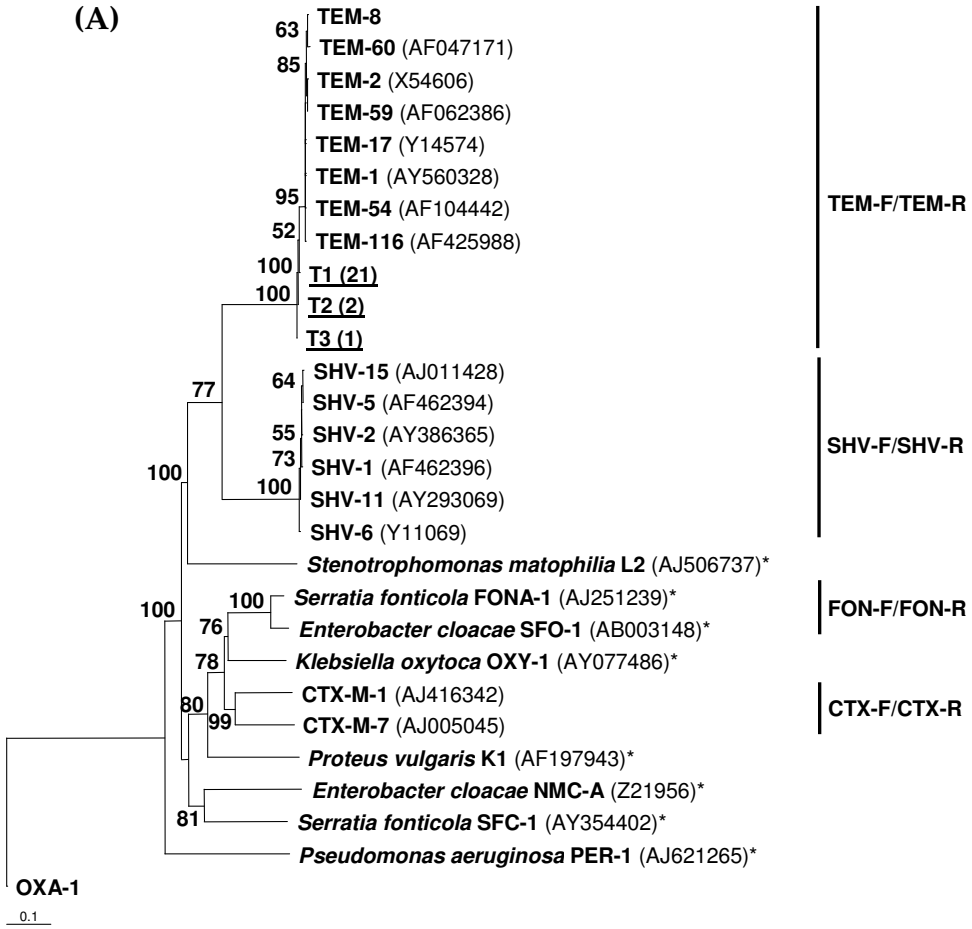

Figure 5.4 Phylogenetic placement of β-lactamase genes belonging to Ambler classes A (Figure 5.3A), B (Figure 5.3B) and D (Figure 5.3C). Trees were constructed using the neighbor-joining method and the class D β-lactamase OXA-1, the subclass B2 β-lactamases Sfh-I, CphA and imiS and the class A β-lactamase TEM-1 gene sequences as outgroups for class A, subclass B1 and class C trees respectively. Bootstrap support values (1000 replicates) above 50 % are shown at nodes. The scale bar indicates 0.1 nucleotide substitution per sequence position. The sets of PCR primers (Table 5.2) targeting β-lactamase genes are shown on the right. Chromosomal genes are indicated with an asterisk. Sequences retrieved during this study are underlined and the number of clones indicated.

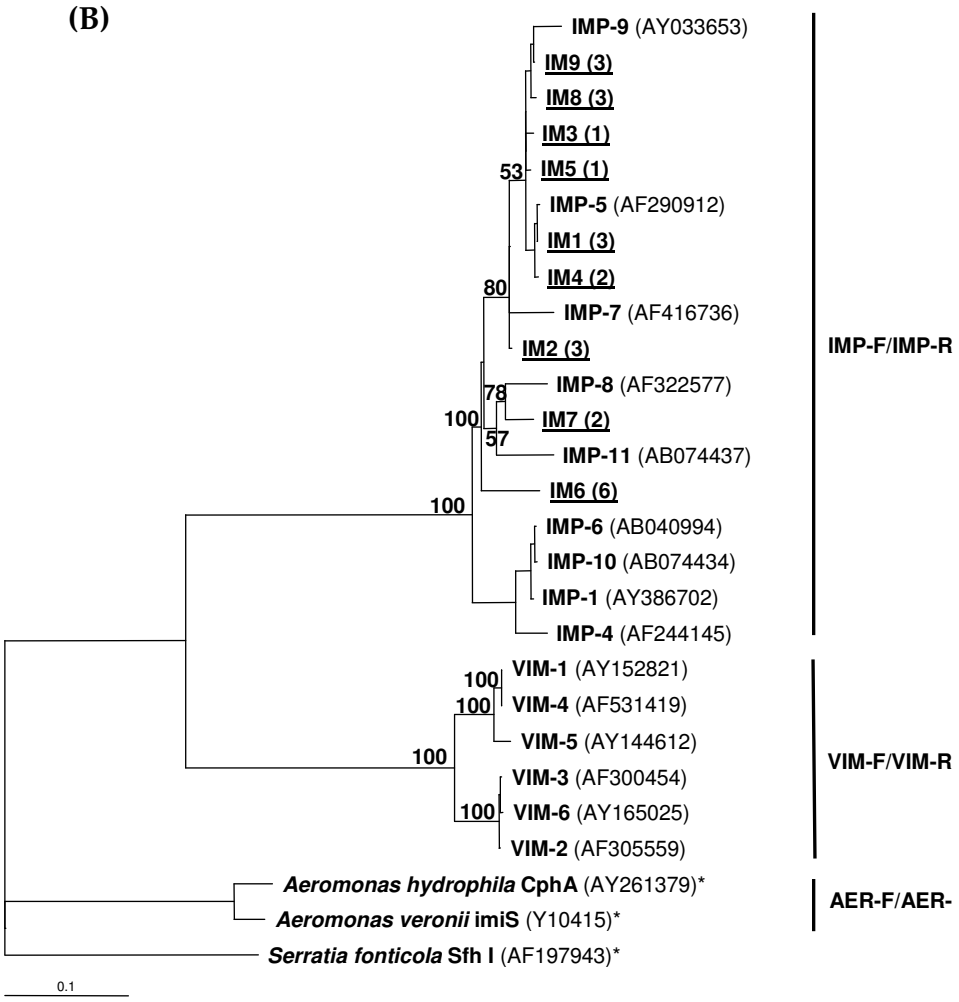

Figure 5.3 Continued

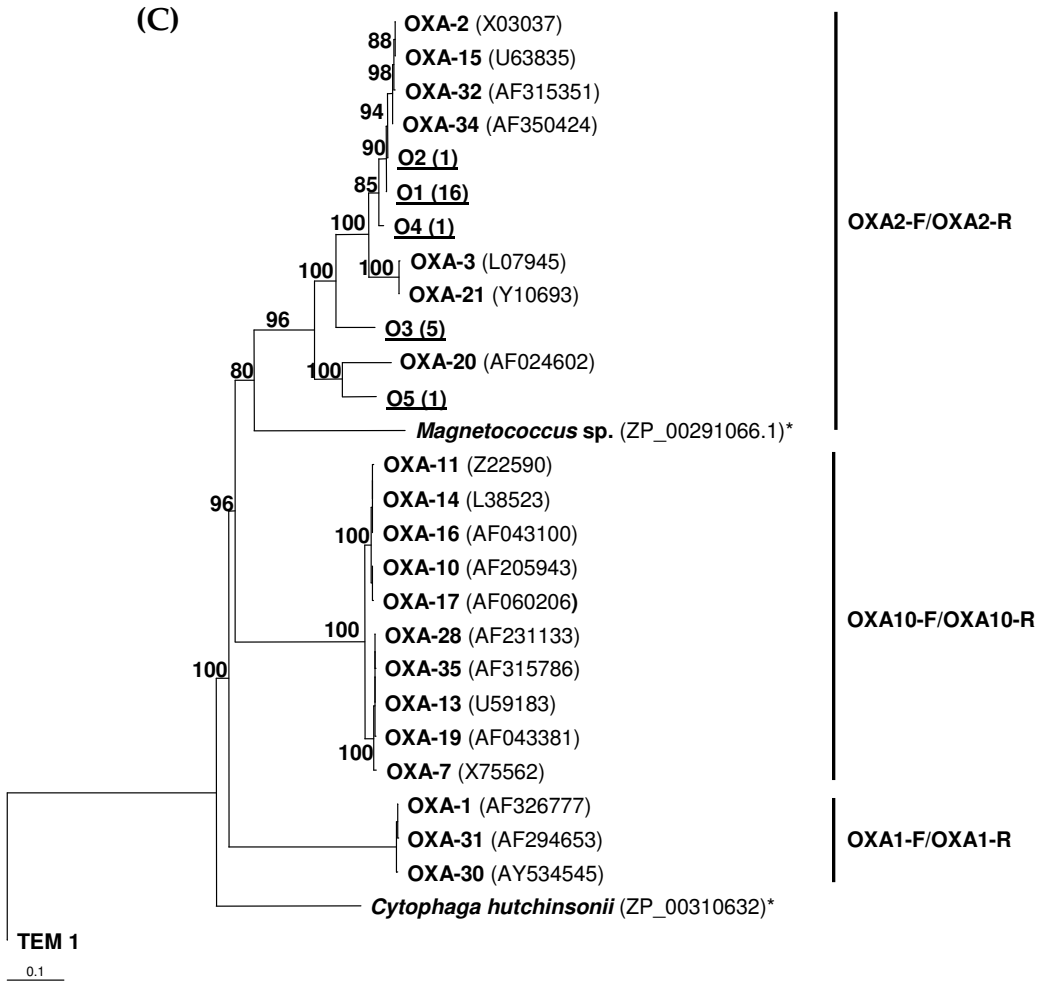

Figure 5.3 Continued

5.4 Discussion

Molecular tools have been successfully applied to study the molecular ecology of genetic determinants of antibiotic resistance other than β-lactamase genes (Aminov *et al.*, 2001; Aminov *et al.*, 2002; Chee-Sanford *et al.*, 2001; Heuer *et al.*, 2002; Schwartz *et al.*, 2003). This work attempted to detect β-lactamase like sequences in environmental bacterial assemblages and describe their molecular diversity. The use of a DNA-based approach has the advantage of covering both the cultivable and non-cultivable bacterial fractions. However, we are aware that the approach applied here detected DNA fragments that may not represent functional β-lactamases. In fact, the DNA sequences retrieved could be part of functional expressed β-lactamase genes, parts of non-functional genes, or parts of functional genes that encode β-lactamase-like proteins that display little or no β-lactamase activity.

Bacterioplankton communities of the lagunar system *Ria de Aveiro* display a phylogenetic composition similar to that found in other coastal and estuarine environments (**Chapter 3**). The lagoon is also subjected to the effect of several human activities. For those two facts, we considered this environment to be a good model for studying the prevalence and molecular diversity of genes that might represent a potential risk for human health.

We decided to limit the search to *bla* sequences encoding enzymes of three of the four established classes of β-lactamases (A, B and D according to Ambler classification), giving special attention to enzymes frequently found in clinical isolates and usually encoded in mobile genetic elements. The primer pairs established during this study, as well as the previously published primer pairs are highly specific. According to the results revealed by specific hybridisation probes, no significant unspecific amplification occurred from DNA extracted from either pure cultures or environmental samples.

DNA elements representing sequences of five of the ten types of β-lactamases under study were present in the three sampling sites. The exceptions were the CTX-M type that is completely absent, OXA-A and OXA-C that could not be detected at the mouth of the estuary, VIM sequences that were absent from site I-6 and SHV, not detected on samples from site I-10. In site N-1 an amplicon representing SHV sequences was only detected after hybridisation with a specific probe. The salinity of this sector of the lagoon

must play an important role in the selection of some bacterial groups that act as reservoirs of this type of genes.

CTX-M β-lactamases seem to have evolved recently: in fact the first report for this type of enzyme is from 1986 and until 1995 a few reports on variants were published. Abruptly after 1995, the number of antibiotic resistance events related to CTX-M like genes has grown-up. This fact and our results are indications that the genes encoding these β-lactam hydrolytic enzymes evolved recently and are restricted to strains circulating in clinical environments.

Surprisingly, even β-lactamase determinants known to confer resistance to carbapenems, a group of β-lactams used as "last-resort" agents in the treatment of infections caused by multiresistant pathogen strains (Norrby, 1995; Rasmussen & Bush, 1997), were detected in the three sampling sites. That is the case of sequences that represent β-lactamase of the group B (the metallo-β-lactamases) like the CphA/IMIS, IMP and VIM types.

The prevalence of β–lactamase encoding sequences seems to be larger in the inner and middle estuarine waters than in the mouth of the estuary. Taking into consideration band intensity, most of the *bla* representing sequences seem to be more abundant in the I-10 site and less abundant in site N-1. All the PCR reactions were performed using equal amounts of environmental DNA, but frequently more intense bands were observed for amplicons obtained when DNA from inner and middle estuarine waters was used as PCR substrate. This was found using two different DNA extraction methods and in triplicates of the PCR reactions. Although the hypothesis of differential efficiency of PCR reactions cannot be excluded, a higher incidence of β-lactamase determinants in the inner and middle sections seems plausible. In fact, those sections are subjected to several polluting sources that might be expected to impact the resistance gene pool, namely aquaculture ponds, diffuse domestic sewage inputs and run-off from agriculture fields (Cunha *et al.*, 2000). It has been reported that unused or un-metabolised therapeutic drugs resulting from human or veterinary medicine are often passed into the aquatic environment in wastewater or run-offs from agriculture fields (Alonso *et al.*, 2001; Chee-Sanford *et al.*, 2001; Kümmerer, 2003; Schwartz *et al.*, 2003). Additionally, antimicrobial agents used in aquacultures are often released directly into the surrounding water (Alonso *et al.*, 2001; Kümmerer, 2003). Studies focusing other antibiotics also reported that the presence of antibiotic resistance determinants in the environment seems to be related to higher levels

of pollution (Aminov *et al*., 2001; Aminov *et al*., 2002; Chee-Sanford *et al*., 2001; Schwartz *et al*., 2003).

In order to assess the molecular diversity of DNA sequences from environmental origin, amplicons obtained with TEM, IMP and OXA-B primers were cloned. In this way we were able to analyse the behaviour of individual products of amplification on denaturing gradient gels. Determination of the base composition of the inserts showed that, as expected, co-migrating bands correspond to 100 % identical molecules and different degrees of retardation on DGGE gels reflect differences in base composition. This result confirms the reliability of the method and the usefulness of PCR-DGGE analysis to estimate levels of genetic diversity within groups of β-lactamase determinants. Analysis of the obtained nucleotide sequences revealed a high percent similarity within each group and in general, high percent similarities with β-lactamase DNA sequences previously characterised from clinical isolates (Dale *et al*., 1985; da Silva *et al*., 2002; de Champs *et al*., 2002; Naas *et al*., 1998; Nucken *et al*., 1989; Riccio *et al*., 2000). However, amino acid substitutions never described for IMP, TEM and OXA-B derivatives were found.

Phylogenetic analysis indicates that the TEM related sequences retrieved during this study constitute a sub-cluster that diverged before the other sequences included in the TEM clade. In contrast, environmental IMP and OXA-B related sequences appear widely distributed within the correspondent clusters. In addition, we found low levels of molecular diversity among the dominant populations of TEM related sequences (known to be highly diverse in clinical environments) and higher levels within the IMP and OXA-B dominant encoding sequences (families that include a lower number of described molecular variants) (Hall & Barlow, 2004). These findings reinforce the hypothesis that the environmental β-lactamase gene pool comprises a complex mixture of ancient naturally occurring sequences and sequences that have been introduced or evolved more recently due to selective pressures resulting from human activities. Also, as stated before, there is evidence of horizontal transfer of β-lactamase genes (Hall & Barlow, 2004): most of the TEM, IMP and OXA-B sequences previously characterised are encoded on plasmids.

The estuarine system studied here constitutes a reservoir of *bla* sequences, not only in terms of presence of genes encoding different classes of β-lactamases but also in what concerns molecular variants of representatives of each class. It is plausible that introduced bacteria are contributing to the diversity of putative *bla* sequences found in *Ria de Aveiro*. However, the hypothesis that the introduction of unused or un-metabolised therapeutic

drugs resulting from human or veterinary medicine and also from aquaculture plants is driving the evolution of antibiotic resistance genetic determinants in the local bacterial community cannot be discarded.

The results presented concern samples collected on a limited number of sites and do not provide information on how stable the pattern of molecular diversity is in a time scale. However, the approach used constitutes a valuable basis to develop more extensive studies. Relevant questions arising from this study are, which factors drive the evolution of *bla* genes in natural environments and what future risks may emerge from this molecular evolution. A more extensive investigation of other ecosystems is needed. Nonetheless, the features revealed by the diversity patterns obtained indicate that monitoring resistance genes in the environment must be carried out at two different levels: detection of gene sequences and analysis of sequence diversity within groups. This last task can be accomplished efficiently and rapidly by combining PCR and DGGE. In fact, evaluation of molecular diversity by DGGE is less expensive and more feasible than DNA sequencing when a large number of samples are examined.

The strategy employed here has been shown to be useful in demonstrating the presence and diversity of β-lactamase genes and can easily be applied to other environments or food products. However, we are aware of the fact that the PCR approach only gives information on molecular variants of sequences already known. Quantitative approaches like those using Real-Time PCR are needed in order to quantify the presence of each sequence type in each sampling site. Other approaches, like environmental metagenome cloning, could help in the task of cataloguing the entire repertoire of antibiotic resistance genes. Also the application of exogenous isolation techniques could allow us to evaluate the transference potential of the β-lactamase genes present in this environment and to characterise the resistance phenotype conferred by those genes.

6. G*enotyping and identification of ampicillin-resistant isolates from estuarine waters*

6.1 Introduction

The four previous chapters presented results obtained by means of methodologies that are independent of the isolation and cultivation of bacteria in the laboratory. In Chapters 3 and 4 the research work was targeted to characterisation of phylogenetic composition and dynamics of bacterioplankton communities in *Ria de Aveiro*; in Chapter 5, the occurrence and molecular diversity of DNA sequences putatively encoding β-lactamases within the estuary were investigated.

Despite the well known and previously referred advantages of culture-independent methodologies, in some cases the link between phylogenetic and functional aspects cannot be established using such techniques. Consequently, cultivation-dependent approaches are useful as a complementary strategy in order to obtain a broader and more realistic picture of phylogeny versus function in a given community (Head *et al*., 1998). In the context of the present work, we considered that a cultivation-dependent strategy would be particularly helpful to complement the results presented in chapter 5, regarding the occurrence and molecular diversity of antibiotic-resistance genetic determinants, namely β-lactamase genes, within the estuary *Ria de Aveiro*. Results obtained are exposed in the present chapter as well as in Chapter 7.

During the study described in this chapter, *Aeromonas*, *Pseudomonas* and *Enterobacteriaceae* isolates were obtained from water samples from *Ria de Aveiro*. Members of the *Enterobacteriaceae* family are normally used as indicators of faecal pollution, since they are introduced in aquatic environments mainly due to anthropogenic activities, and are commonly associated with infectious diseases (Goñi-Urriza *et al*., 2000). *Aeromonas* and *Pseudomonas* isolates were also assessed since they constitute main components of the cultivable fraction of bacterioplankton communities from aquatic environments, and so if the environmental genetic pool is to be characterized those groups must be considered (Anzai *et al*., 2000; Gõni-Urriza *et al*., 2000). Moreover, the production of several β-lactamases by *Enterobacteriaceae*, *Aeromonas* and *Pseudomonas* isolates has been documented by several authors (Livermore *et al*., 1995; Massidda *et al*., 1991).

Among several genotyping techniques previously described and extensively applied, repetitive extragenic palindromic elements-polymerase chain reaction (REP-PCR) has been reported to be sufficiently reliable, rapid and sensitive to distinguish between a

broad range of bacterial genera and species (Versalovic *et al*., 1991). REP-PCR is a fingerprinting method that produces specific band patterns for each strain as a result of the amplification of repetitive DNA elements present in bacterial genomes. REP-PCR has most frequently been applied in combination with other typing methods, namely ERIC- and BOX-PCR (Alves *et al*., 2002; Alves *et al*., 2004; Versalovic *et al*., 1991), but it has also been employed and suggested for independent use (Borges *et al*., 2003). The successful application of REP-PCR to discriminate *Enterobacteriaceae*, *Aeromonas* and *Pseudomonas* strains has been reported (Bhattacharya *et al*., 2003; Borges *et al*., 2003; Szczuka & Kaznowski, 2004).

DNA sequencing of conserved regions within phylogenetic markers, such as the 16S rRNA gene, is a promising resource to identify bacterial isolates. Broad-range PCR primers have been described targeting highly conserved regions of the 16S rRNA gene from a variety of microorganisms (Giovanonni *et al*., 1988). Highly variable regions within the amplified fragment allow phylogenetic analysis and frequently species-level identification (van de Peer *et al*., 1996). Ribosomal RNA-based approaches have increasingly and successfully been applied to identify *Enterobacteriaceae*, *Pseudomonas* and *Aeromonas* isolates.

The aim of the study presented in this chapter was to characterise the genetic diversity and phylogenetic affiliation of Gram-negative ampicillin-resistant isolates obtained from *Ria de Aveiro* water samples using 16S rDNA sequence analysis, phenotypic methods and REP-PCR.

6.2 Materials and methods

6.2.1 Sampling

Samples were collected from the same three sites sampled during the study presented in Chapter 5 (named N-1, I-6 and I-10), previously characterised in what concerns the molecular diversity and dynamics of the resident microbial communities, and the occurrence and molecular diversity of DNA sequences putatively encoding β-lactamases (**Chapter 3**, **Chapter 4** and **Chapter 5;** Figure 2.1). Sampling procedures were as described in Chapter 5.

6.2.2 Isolation of ampicillin-resistant colonies

Water samples were filtered through 0.45-µm-pore-size cellulose ester filters (Pall Life Sciences, Michigan, USA), and the membranes placed onto GSP-agar or MacConkey-agar plates (Merck, Darmstadt, Germany) supplemented with 50 µg/ml of ampicillin to select for potential β-lactamase producers. Plates were incubated at 30 ºC (GSP plates) or 37 ºC (MacConkey plates) for 16 h. Individual ampicillin-resistant colonies were purified and stored in 20 % glycerol at -80 ºC.

6.2.3 DNA extraction

DNA was isolated from 2 ml of liquid cultures harvested at late exponential phase, with the Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania). To improve lysis a previous step of incubation with lysozyme was performed. Purified DNA was aliquoted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -20 ºC. DNA concentrations were estimated spectrophotometrically.

6.2.4 REP-PCR genomic fingerprinting

For genotyping, repetitive extragenic palindromic PCR (REP-PCR) was performed on total DNA with primers REP1R (5′-IIIICGICGICATCIGGC-3′) and REP2I (5′-ICGICTTATCIGGCCTAC-3′). DNA amplification was carried out in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Richmond, CA, USA) with reagents from MBI Fermentas (Vilnius, Lituânia). The reaction mixture consisted of 1x PCR buffer (PCR buffer with $(NH_4)_2SO_4$), 3 mM $MgCl_2$, 5 % dimethylsulfoxide, 200 µM each nucleotide, 2 µM each primer, 1 U *Taq* polymerase and 50-100 ng purified template DNA made up to 25 µl with sterile distilled water. The reaction mixture was denatured for 7 min at 94°C and then subjected to 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min 40°C, extension for 8 min at 65°C, and a final extension for 16 min at 65°C (Versalovic *et al*., 1991).

6.2.5 Electrophoresis and computer analysis

The PCR products underwent 1.5 % agarose gel electrophoresis for 2.45 h under constant 80 V in 1X Tris–acetate–EDTA buffer. The DNA Ladder MIX (MBI, Fermentas) was run at both sides of each gel. Gel images were acquired with the Molecular Imager FX™ system (Bio-Rad Laboratories, Richmond, CA, USA). The genomic fingerprints obtained were compared for similarity by visual inspection of band patterns and were further processed using the GelCompar II (Applied Maths, Kortrijk, Belgium) software. Similarity matrices were calculated with the Dice coefficient. Cluster analysis of similarity matrices was performed by the unweighted pair-group method using arithmetic averages (UPGMA). Isolates representing distinct REP patterns were selected for subsequent identification.

6.2.6 Sequencing for taxonomical identification

Strains were identified by partial sequencing of the 16S rDNA. The universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'- GGTTACCTTGTTAC GACTT-3') (Lane, 1991) were used to amplify nearly full-length 16S rRNA gene. A PCR profile with an initial denaturation of 3 min at 93 ºC, 35 cycles of 94 ºC for 1 min, 51 ºC for 2 min, and 72 ºC for 2 min and a final extension cycle at 72 ºC for 10 min was applied. The PCR mixtures had the following composition: 25 µL reaction mixtures contained 1X PCR buffer (PCR buffer with $(NH_4)_2SO_4$), 3 mM $MgCl_2$, 5 % dimethylsulfoxide, 100 µM each nucleotide, 7.5 pmol of each primer, 0.5 U of *Taq* polymerase, and 50-100 ng of purified DNA. PCR reactions were carried out in an iCycler Thermal Cycler (Bio-Rad Laboratories, Richmond, CA, USA) using *Taq* polymerase, nucleotides and buffers purchased from MBI Fermentas (Vilnius, Lithuania).

Both primers were used for the sequencing reactions. PCR products were purified with the JETQUICK PCR Purification Spin Kit (GENOMED, Löhne, Germany) and used as template in the sequencing reactions, carried out using an ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City,

California, USA). The ABI PRISM® 310 Genetic Analyser (PE Applied Biosystems) was employed for fragment separation and sequence analysis.

6.2.7 Phylogenetic analysis

Edited sequences were compared to the GenBank nucleotide data library using the BLAST software at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) in order to determine their closest phylogenetic relatives (Altschul *et al*., 1997). Sequence alignments were performed using the software Clustal X (Thompson *et al*., 1997). Phylogenetic analyses were performed with PAUP (Swofford, 1999). Rooted trees were constructed using the neighbour-joining method based on about 1000 nucleotides. Bootstrap values (1000 replicates) were determined.

6.2.7 Biochemical tests

Identification of all isolates affiliated with genera *Escherichia* or *Shigella* was confirmed using API20E test strips (Biomerieux, Marcy-L'Etoile, France). Colonies were re-suspended in 0.85 % sterile saline solution to produce visible turbidity equivalent to a 1.0 McFarland standard. Samples of this suspension were used to inoculate the 20 reaction chambers of an API 20E unit according to the manufacturer instructions. The test chambers for arginine dihydrolase, lysine decarboxylase, omithine decarboxylase, and urease were overlaid with sterile mineral oil, and the unit was incubated for 24 h at 37 °C. Thereafter, the following reagents were added to the respective chambers: 1 drop of 40 % KOH and 1 drop of 6 % alpha-naphthol for the Voges- Proskauer test; 1 drop of ferric chloride for tryptophan deaminase; 1 drop of Kovacs reagent for the indole test. The resulting combination of colour reactions in the 20 chambers was converted into a seven-digit octal code for each organism, and identification of each organism was determined by comparing the code with entries in a manual provided by the manufacturer.

6.3 Results

6.3.1 REP-PCR genomic fingerprinting

A total of 164 isolates were selected from plates supplemented with ampicillin and subjected to genotyping using REP-PCR. *Enterobacteriaceae*, *Aeromonas* and *Pseudomonas* isolates generated complex banding patterns. Occasionally the same pattern was observed for isolates selected from MacConkey and GSP plates from the same sampling site. Only rarely the same pattern was detected in different sampling sites. The number of bands obtained ranged between 12 and 25 bands, with an average of 16 bands per isolate. The bands were uniformly distributed between 100 bp and 5 Kb. Patterns complexity and band sizes were not significantly different between isolates included in the three phylogenetic groups considered in this study.

Gel images were analysed using the GelCompar II (Applied Maths, Kortrijk, Belgium) software. Considering a cut-off value of 80 % (as suggested by Cho & Tiedje, 2000) a total of 124 different REP types were distinguished, from which only 33 were represented by more than one isolate. The 4 dendrograms obtained are shown in Figure 6.1. Similarities between the most distantly related isolates were 34 % for the *Enterobacteriaceae* isolates (excluding *Escherichia* and *Shigella* isolates), 40 % for *Escherichia/Shigella* isolates, 36 % for *Pseudomonas* isolates and 32 % for *Aeromonas* isolates. Generally, the isolates of the 3 sampling sites were uniformly distributed along the dendrograms however some putative site-dependent clusters could be identified (Figure 6.1).

In the cases of the *Enterobacteriaceae* (except *Escherichia* and *Shigella*) dendrogram (Figure 6.1A), the *Pseudomonas* dendrogram (Figure 6.1B) and the *Aeromonas* dendrogram (Figure 6.1C) the isolates distribution was generally consistent with phylogenetic affiliations obtained by 16S rDNA sequence analysis (different species clustered separately). However some incongruences could be identified, namely isolates identified as *Aeromonas hydrophila*, *Aeromonas salmonicida* and *Aeromonas veronii* were not always included in the same REP clusters.

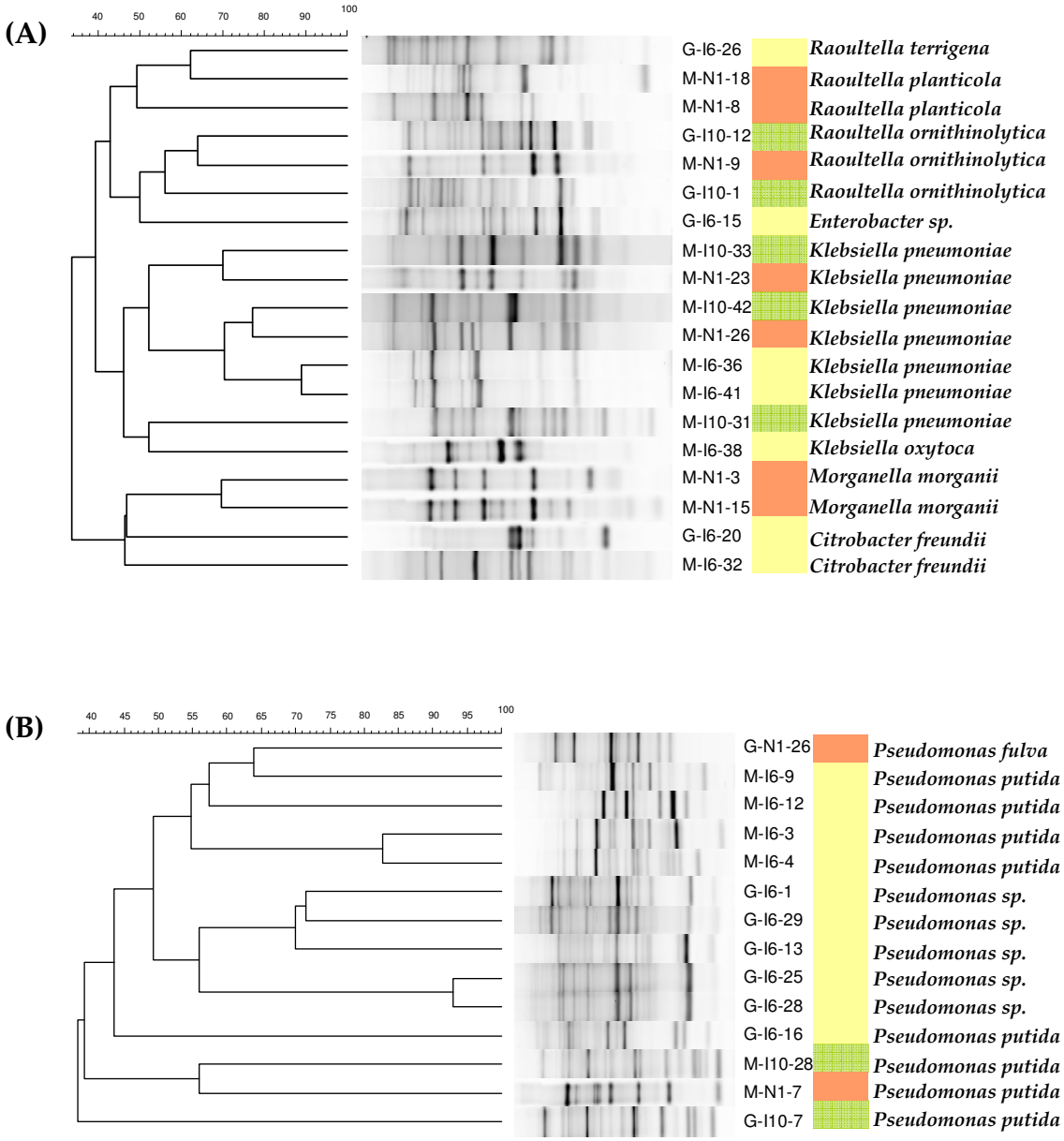

Figure 6.1 Dendrograms based on UPGMA cluster analysis of Dice similarity values between REP-PCR fingerprint patterns from isolates identified as *Enterobacteriaceae* (except those identified as *Escherichia* or *Shigella*) (A), *Pseudomonas* (B), *Aeromonas* (C) and *Escherichia/Shigella* (D). The phylogenetic affiliations presented were obtained by 16S rDNA sequence analysis. Isolates were collected from site N-1 (■), I-6 (■) and I-10 (■).

(C)

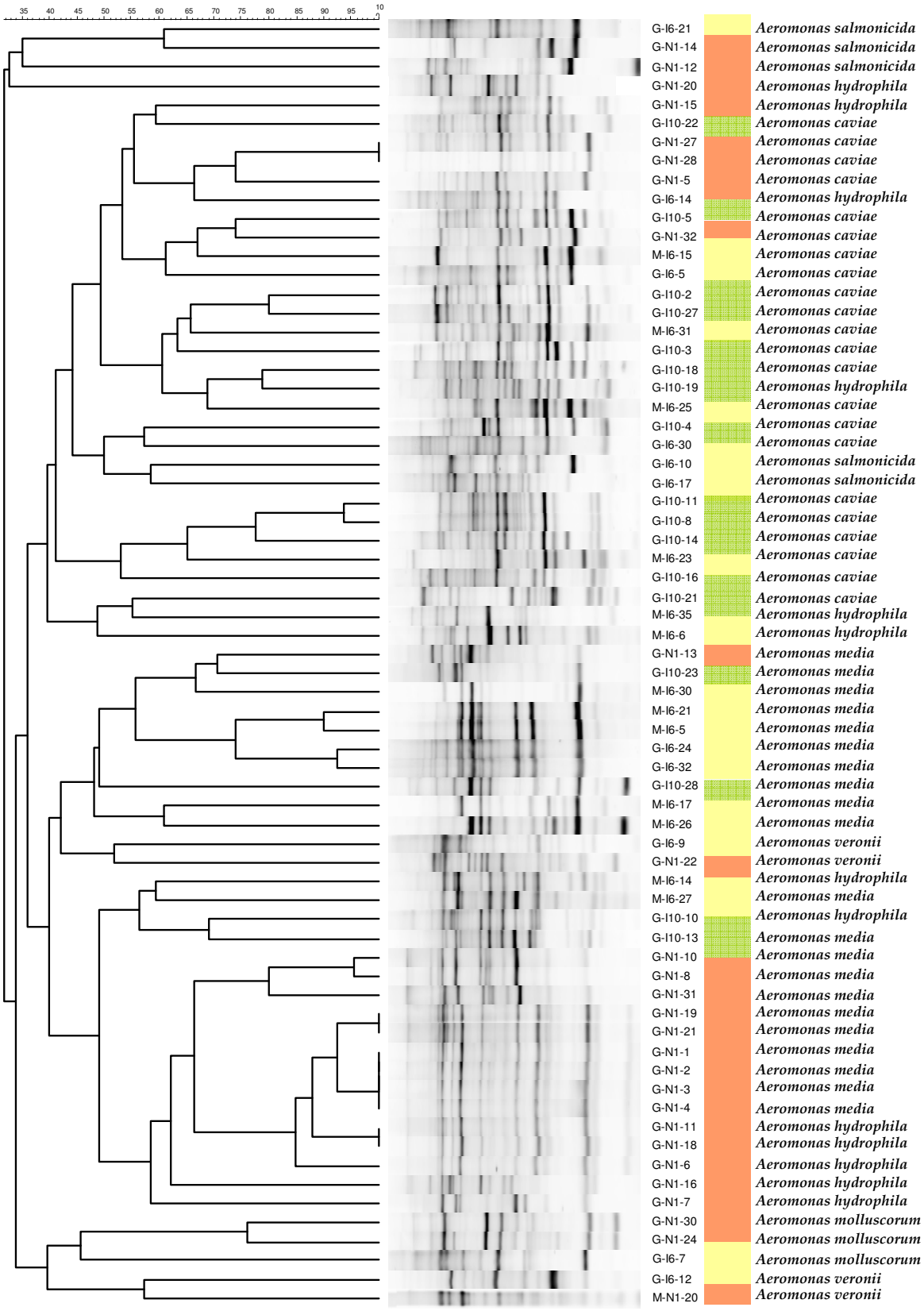

Figure 6.1 Continued.

(D)

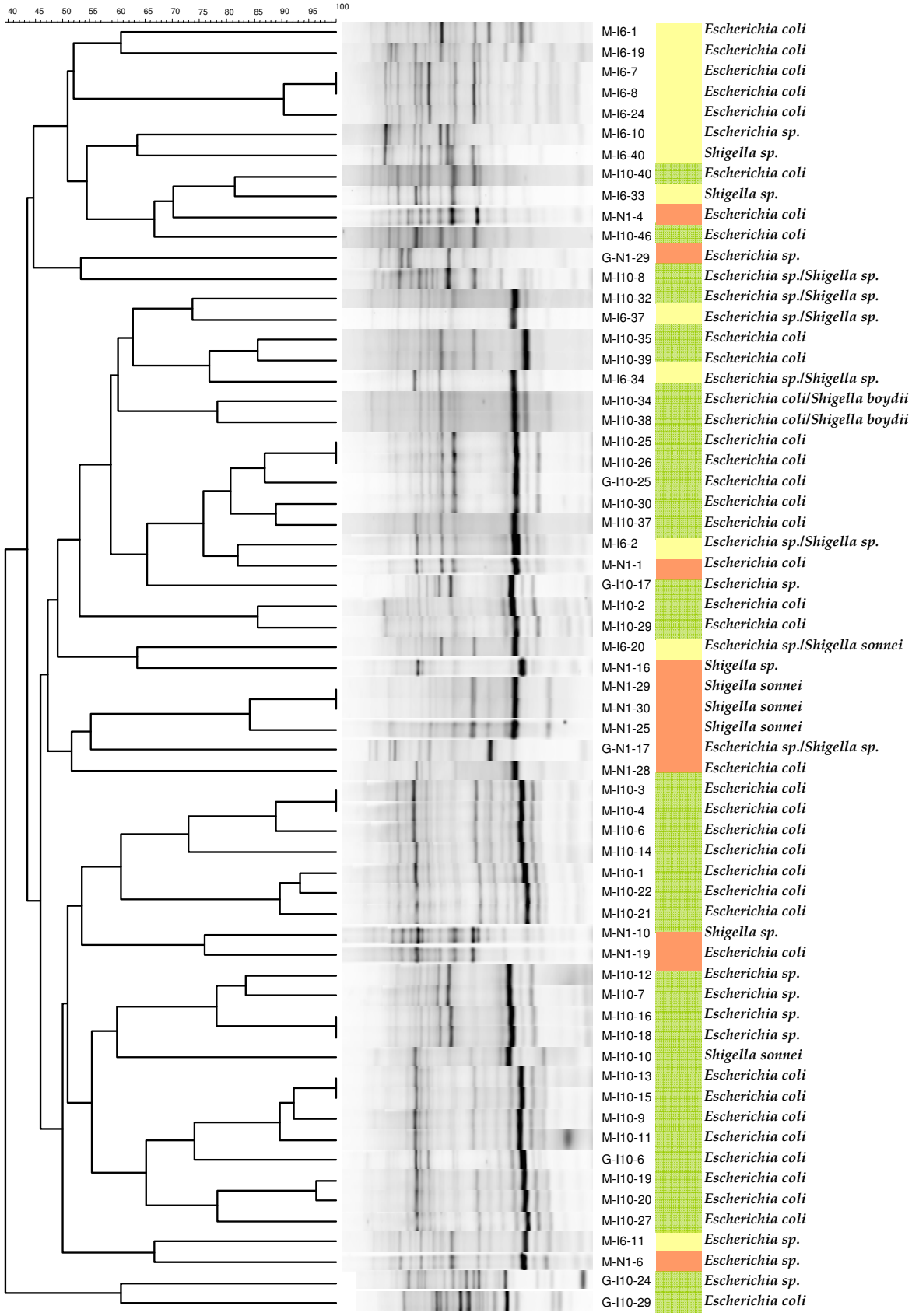

Figure 6.1 Continued.

6.3.2 16S rDNA based phylogenetic affiliation of isolates

From each of the 124 REP types identified, one isolate was selected for further analysis, corresponding to 54 isolates from MacConkey plates and 70 isolates from GSP plates. Nearly-full length of the 16S rRNA gene was amplified and the nucleotide sequence of approximately 1000 bp was obtained. After being edited, sequences were compared with the GenBank database by using the BLAST software. In figure 6.1 the phylogenetic affiliation of the closest relatives is presented. Isolates were identified as *Aeromonas* sp. (57 isolates), *Pseudomonas* sp. (13 isolates), *Klebsiella* sp. (7 isolates), *Raoultella* sp. (6 isolates), *Citrobacter* sp. (2 isolates), *Morganella* sp. (2 isolates) and *Enterobacter* sp. (1 isolate). Due to difficulties in distinguishing *Escherichia* and *Shigella* isolates based solely in the 16S rDNA sequence analysis, 33 isolates included in those genera were subsequently re-identified using API 20E test strips (results will be presented in section 6.3.3 of the present chapter).

Phylogenetic analysis of the retrieved sequences allowed the construction of two phylogenetic trees presented in Figure 6.2, which also comprise previously reported 16S rRNA gene sequences from representatives of several species included in the genera retrieved during this study. As expected all *Enterobacteriaceae* genera constitute clearly separated clusters except for *Escherichia* and *Shigella* groups which could not be undoubtedly separated. *Aeromonas* and *Pseudomonas* clusters are only distantly related. Within the *Aeromonas* cluster there is no clear separation between the considered species.

In general isolates recovered from sites I-10, I-6 and N-1 were widely distributed within the phylogenetic trees. Exceptions were isolates affiliated with genus *Morganella* (only retrieved from site N-1), isolates affiliated with genera *Enterobacter* and *Citrobacter* (only retrieved from site I-6) and isolates affiliated with genus *Pseudomonas* (most frequently isolated from site I-6).

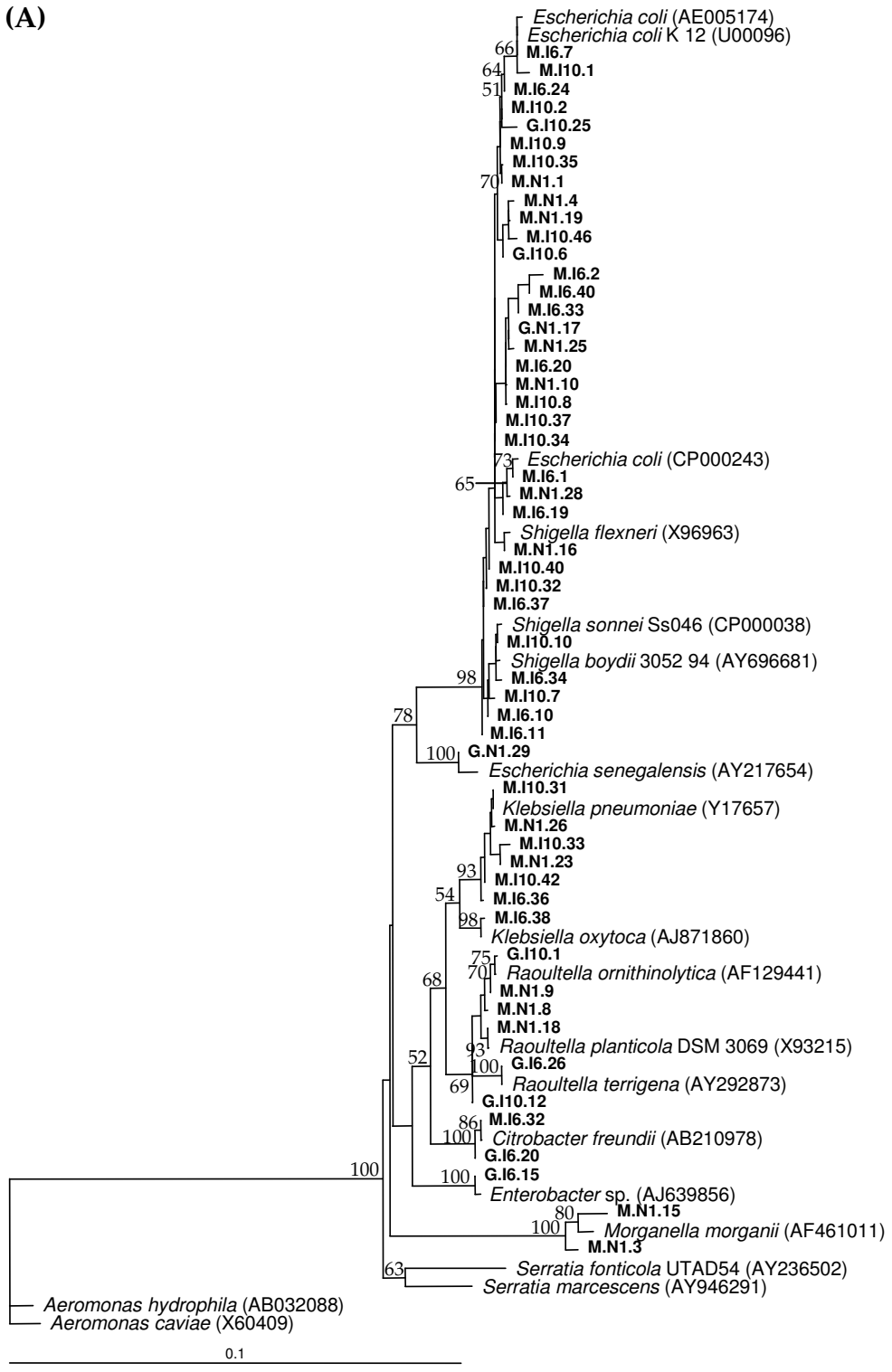

Figure 6.2 Phylogenetic relationships between sequences retrieved during this study and reference taxa: (A) *Enterobacteriaceae,* (B) *Aeromonas and Pseudomonas*. The trees were constructed based on 1000 bp. The 16S rRNA gene sequences of *Aeromonas hydrophila* and *Aeromonas caviae* were used as outgroup in the construction of tree A and the 16S rRNA gene sequences of *Escherichia coli* and *Shigella boydii* were used as outgroup in the construction of tree B. Bootstrap support values (1000 replicates) above 50 % are shown at nodes.

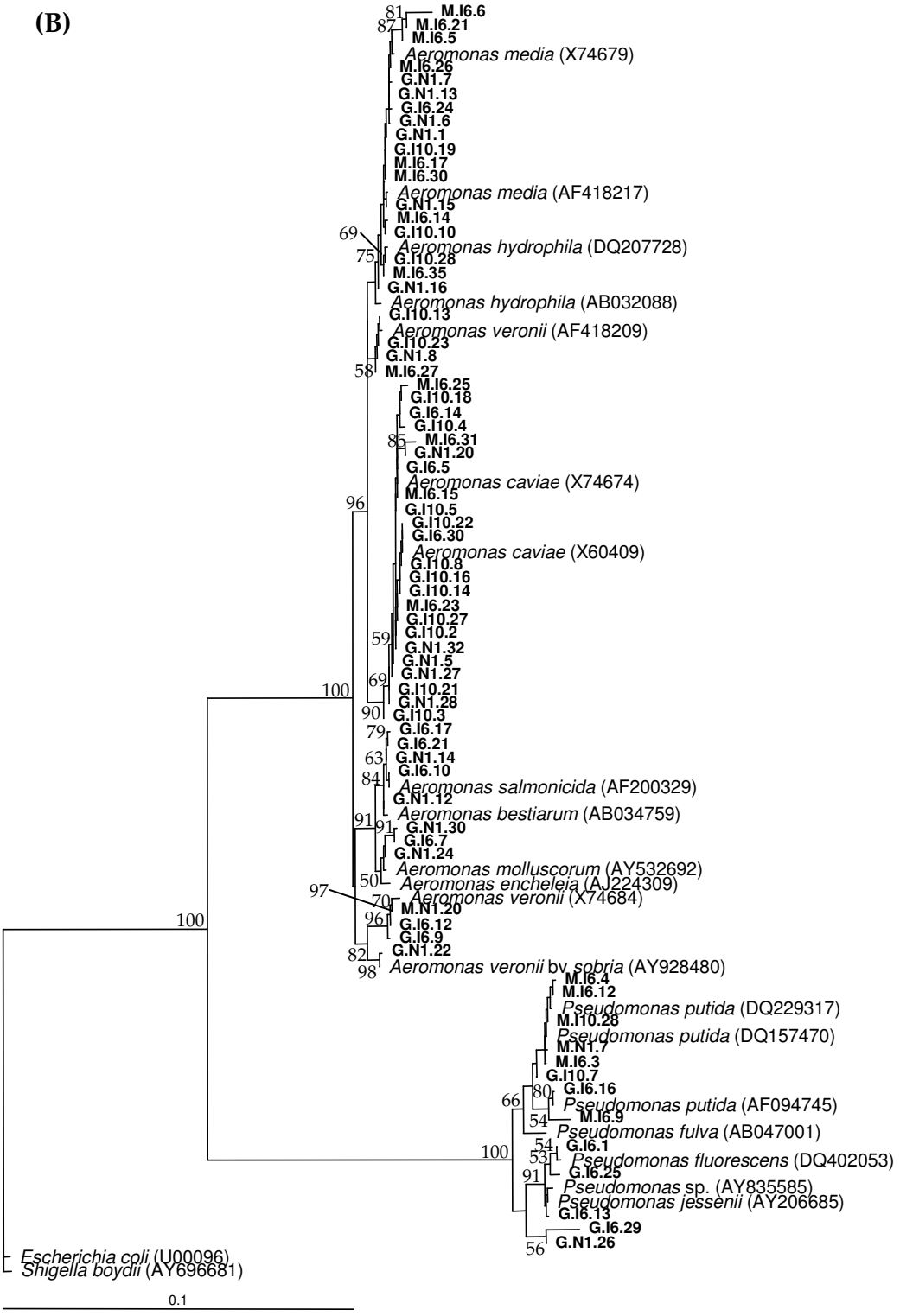

Figure 6.2 Continued.

6.3.3 Identification of Escherichia/Shigella isolates by biochemical tests

The identification of 33 isolates affiliated with genera *Escherichia* or *Shigella* based on 16S rDNA sequence analysis was confirmed using API 20E test strips. Results are shown in Table 6.1.

Table 6.1 Results obtained from 16S rDNA and API 20E based identification of *Escherichia*/*Shigella* isolates.

Isolate identification	16S rDNA closest relative (% similarity)	API 20E result (% identification[a])
M.I10.1	*Escherichia coli* (100 %)	*Escherichia coli* (94.8 %)
M.I10.2	*Escherichia coli* (99 %)	*Escherichia coli* (99.8 %)
M.I10.7	*Escherichia sp.* (99 %)	*Escherichia coli* (99.8 %)
M.I10.8	*Shigella* sp. / *Escherichia* sp. (99 %)	*Escherichia coli* (99.8 %)
M.I10.9	*Escherichia coli* (100 %)	*Escherichia coli* (96.1 %)
M.I10.10	*Shigella sonnei* (99 %)	*Escherichia coli* (98.1 %)
M.I10.32	*Shigella* sp. /*Escherichia sp.* (99 %)	*Escherichia coli* (80.6 %)
M.I10.34	*Shigella boydii* / *Escherichia coli* (99 %)	*Escherichia coli* (99.9 %)
M.I10.35	*Escherichia coli* (100 %)	*Escherichia coli* (97.7 %)
M.I10.37	*Escherichia coli* (99 %)	*Escherichia coli* (96.4 %)
M.I10.40	*Escherichia coli* (99 %)	*Escherichia coli* (99.6 %)
M.I10.46	*Escherichia coli* (99 %)	*Escherichia coli* (99.5 %)
G.I10.6	*Escherichia coli* (99 %)	*Escherichia coli* (96.1 %)
G.I10.25	*Escherichia coli* (99 %)	*Escherichia coli* (99.9 %)
M.I6.1	*Escherichia coli* (99 %)	*Escherichia coli* (99.6 %)
M.I6.2	*Shigella* sp. / *Escherichia* sp. (99 %)	*Escherichia coli* (85.6 %)
M.I6.7	*Escherichia coli* (100 %)	*Escherichia coli* (99.6 %)
M.I6.10	*Escherichia sp.* (100 %)	*Escherichia coli* (99.6 %)
M.I6.11	*Escherichia sp.* (100 %)	*Escherichia coli* (99.8 %)
M.I6.19	*Escherichia coli* (99 %)	*Escherichia coli* (99.9 %)
M.I6.20	*Escherichia sp. / Shigella sonnei* (99 %)	*Escherichia coli* (97.7 %)
M.I6.24	*Escherichia coli* (100 %)	*Escherichia coli* (98.4 %)
M.I6.33	*Shigella* sp. (99 %)	*Escherichia coli* (86.2 %)
M.I6.34	*Shigella* sp. / *Escherichia sp.* (99 %)	*Escherichia coli* (98.9 %)
M.I6.37	*Shigella* sp. / *Escherichia sp.* (99 %)	*Escherichia coli* (99.8 %)
M.I6.40	*Shigella* sp. (100 %)	*Escherichia coli* (89.6 %)
M.N1.1	*Escherichia coli* (100 %)	*Escherichia coli* (99.8 %)
M.N1.4	*Escherichia coli* (99 %)	*Escherichia coli* (99.6 %)
M.N1.6	*Escherichia sp.* (100 %)	*Escherichia coli* (99.8 %)
M.N1.10	*Shigella* sp. (99 %)	*Escherichia coli* (99.8 %)
M.N1.16	*Shigella sp.* (100 %)	*Escherichia coli* (96.4 %)
M.N1.19	*Escherichia coli* (99 %)	*Escherichia coli* (99.6 %)
M.N1.25	*Shigella sonnei* (99 %)	*Escherichia coli* (99.9 %)
M.N1.28	*Escherichia coli* (100 %)	*Escherichia coli* (99.9 %)
G.N1.17	*Shigella* sp. / *Escherichia* sp. (99 %)	*Escherichia coli* (99.7 %)

[a]represents an estimate of how closely the profile corresponds to the taxon relative to all the other taxa in the database.

6.4 Discussion

In the study presented here 164 ampicillin-resistant Gram-negative isolates were obtained from 3 sampling sites in the estuary *Ria de Aveiro*. The genotypic diversity of the selected isolates was evaluated by using REP-PCR. This method yielded highly reproducible and complex band patterns, revealing high capacity to discriminate isolates. However, the capacity to provide information about the phylogeny of the isolates was found limited in some cases, namely when considering isolates included in the genus *Aeromonas*. As reported by other authors the method here applied can be used as an alternative strategy to identify isolates but most of the times in combination with other genotyping methods (Alves *et al*., 2004).

Results obtained revealed a high degree of genetic variability among the isolates retrieved. A cut-off value of 80 % yielded 124 different genotypes, from which 91 unique patterns were represented by only one isolate. This result is in accordance with previously reported studies concerning Gram-negative isolates from aquatic environments (Baldy-Chudzik *et al*., 2001; Borges *et al*., 2003). This high genetic heterogeneity between isolates obtained from natural environments has been attributed to the fact that different sampling sites usually display completely distinct characteristics. This is the case of the sites sampled during this study, which are subjected to contaminations from different polluting sources and also are characterised by different physical features, such as different temperature and salinity values (**Chapter 4**; **Chapter 5**). Additionally, high degrees of genetic variability were also found between isolates retrieved from the same sampling site. This fact may be due to the dynamic characteristics of the estuary, subjected to semi-diurnal tides. Despite the fact that a few putative site-specific clusters have been identified, similarity data between isolates did not enable differentiation between sampling sites.

Identification of isolates displaying different REP-patterns (considering a cut-off value of 80 %) was performed by analysis of 16S rRNA gene sequences (Lane, 1991). This method has been widely accepted and applied for the identification of bacterial isolates from several genera and species (Clarridge III, 2004). However, some constraints were recognized during this study. Firstly, it was impossible to identify at species level several isolates affiliated to genus *Aeromonas*. This impossibility has been recognised by other authors (Yáñez *et al*., 2003) and alternative identification molecular methods have been

proposed (Sen, 2005; Tacão *et al*., 2005b). For the purposes of the study described here we considered that identification at genus level would be sufficient.

Additionally, difficulties were encountered in the case of isolates affiliated with genera *Escherichia* and *Shigella*. In some cases it was not possible to affiliate the isolate to only one of these genera. We complemented the 16S rDNA-based identification with biochemical tests, and obtained some divergent results (isolates identified as *Shigella* using 16S rDNA analysis were identified as *E. coli* using biochemical tests). It has been reported that *Escherichia* and *Shigella* are very closely related genera and may even constitute only one genus (Jin *et al*., 2002; Pupo *et al*., 2000).

Ampicillin-resistant isolates were affiliated to 9 genera, commonly isolated from aquatic environments (Lin & Byiela, 2005; Roe *et al*., 2003; Rosser *et al*.; 1999). In agreement with previously published studies regarding polluted aquatic environments, among the *Enterobacteriaceae*, isolates affiliated with genera *Escherichia/Shigella* were clearly more abundant (Borges *et al*., 2003; Roe *et al*., 2003). Patent differences were not registered between sampling sites.

7. Occurrence and diversity of integrons and β-lactamase genes among ampicillin-resistant isolates from Ria de Aveiro

7.1 Introduction

Besides the well known clinical extensive use of β-lactams, these compounds are also frequently used in sub-therapeutic doses to prevent diseases and promote growth of livestock. A few studies have already been reported concerning the levels of β-lactam resistance and the presence of resistance genetic determinants among environmental isolates. Contamination of aquatic environments is of particular concern since it may contribute to the prevalence of antimicrobial resistant bacteria in humans, acquired directly or indirectly through the human food chain. Results obtained allowed the detection and characterisation of previously described β-lactamases and also enzymes displaying novel catalytic properties (Henriques *et al*., 2004; Poirel *et al*., 2005; Saavedra *et al*., 2003).

The rapid emergence of antibiotic resistance among bacteria is, in a great extent, due to the dissemination of antibiotic resistance genes by horizontal gene transfer, mediated by plasmids, transposons and integrons (Davidson, 1999; Ploy *et al*., 2000; White *et al*., 2001). Integrons are characterised by the presence of an *intI* gene encoding an integrase, a recombination site (*attI*) and a strong promoter. Several studies support the hypothesis that integrons play a major role in the spread of multidrug resistance in Gram-negative bacteria due to their ability to capture gene cassettes from the environment and incorporate them by site-specific recombination (Ploy *et al*., 2000; White *et al*., 2001). Thus, detection and characterisation of integrons containing antibiotic resistance genes are key steps to evaluate the potential of a certain environment to represent a reservoir of antibiotic resistance.

The occurrence and molecular diversity of DNA sequences putatively encoding β-lactamases in *Ria de Aveiro* has previously been assessed using culture-independent methodologies (**Chapter 5**). Sequences representing all families of β-lactamases studied (belonging to Ambler classes A, B and D) were detected. Moreover, nucleotide sequence analysis suggested that this aquatic ecosystem is a reservoir of molecular diverse putative β-lactamase sequences. However the culture-independent methodologies gave no information about the resistance phenotypes conferred by the detected genes or the phylogenetic affiliation of the bacteria carrying those genes. Therefore, the application of complementary culture-dependent approaches is essential to extend our knowledge on

these subjects and to confirm the importance of the referred environment as a reservoir of antibiotic resistance genetic determinants.

The aim of this study was to assess the occurrence and molecular diversity of β-lactamase genes among Gram-negative ampicillin-resistant bacteria isolated from *Ria de Aveiro*, using PCR with specific primers and sequencing analysis of the obtained amplicons. The presence of class 1 and class 2 integrons was investigated; when detected, the identity of the gene cassettes they enclosed was assessed. The antibiotic resistance profiles of the isolates were established, with special emphasis in β-lactam resistance. Taken together, results obtained provide new evidence to clarify the importance of aquatic environments as reservoirs of antibiotic resistance genetic determinants.

7.2 Materials and methods

7.2.1 Bacterial strains used as positive controls

Strains used as positive controls for PCR amplification of β-lactamase genes were described in Chapter 5 (Table 5.1). An isolate obtained during this study, carrying an integron containing the gene CARB-2 was used as positive control for primer set CARB_F/CARB_R. A strain of *Salmonella enterica* serovar *Typhimurium* (kindly provided by Séamus Fanning, University College Dublin, Ireland) and a strain of *Escherichia coli* (kindly provided by John Maurer, University of Georgia, Greece) were used as positive controls for amplifications of class 1 and class 2 integrons, respectively.

7.2.2 Bacterial isolates resistant to ampicillin

Ampicillin-resistant bacterial isolates were previously obtained, subjected to genotyping and identified (**Chapter 6**). During isolation, for each sampling site, membranes were also placed on plates without ampicillin in order to estimate the proportion of ampicillin-resistant colonies (counts were done in triplicate).

7.2.3 Antibiotic susceptibility testing

Antimicrobial resistance patterns were determined by the agar disc diffusion method on Mueller-Hinton agar (Merck, Darmstadt, Germany). Discs containing the following antibacterial agents were used: ampicillin (10 μg), carbenicillin (100 μg), amoxicillin (10 μg), amoxycillin/clavulanic acid (20 μg/10 μg), piperacillin (100 μg), piperacillin/tazobactam (100 μg/10 μg), cephalotin (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), cefepime (30 μg), aztreonam (30 μg), imipenem (10 μg), tetracycline (30 μg), gentamicin (10 μg), amikacin (30 μg), tobramycin (10 μg), ciprofloxacin (5 μg) and sulphamethoxazole/trimethoprim (25 μg) (Oxoid, Basingstoke, UK). After 24 h of incubation at either 37°C (for *Enterobacteriaceae*) or 30°C (for *Aeromonas* and *Pseudomonas*), organisms were classified as sensitive, intermediate, or resistant according to the National Committee for Clinical Laboratory Standards criteria (NCCLS, 2001).

7.2.4 β-lactamase-specific PCRs

Primers used in this study to amplify β-lactamase genes as well as references, annealing temperatures and predicted amplicon sizes were described in Chapter 5 (Table 5.2), except for primers targeting CARB-like β-lactamases (Table 7.1) which were designed during this study following the procedures described in Chapter 5. All primer sets were tested in all isolates except for primers AER-F and AER-R which were tested only on isolates identified as *Aeromonas* sp..

The PCR mixtures used to detect β-lactamase genes in the genomes of ampicillin-resistant isolates had the following composition: 25 μl reaction mixtures contained 1X PCR buffer (PCR buffer with $(NH_4)_2SO_4$), 3 mM $MgCl_2$, 5 % dimethylsulfoxide, 100 μM each nucleotide, 7.5 pmol of each primer, 0.5 U of *Taq* polymerase, and 50-100 ng of purified DNA. The temperature profile was as follows: initial denaturation (94 ºC for 9 min); 30 cycles of denaturation (94 ºC for 30 s), annealing (as indicated in Tables 5.2 and 7.1 for 30 s), and extension (72 ºC for 1 min); and a final extension (72 ºC for 10 min). PCR products (5 μl) were analysed by electrophoresis on a 1.5 % agarose gel and stained with ethidium bromide.

7.2.5 Integron-specific PCRs

PCR conditions for the detection of the class 1 and class 2 integrons and the presence of gene cassettes have been described elsewhere (Goldstein *et al*., 2001; Kraft *et al*., 1986; White *et al*., 2001). Integrase genes were detected using primer sets intI1_F/intI1_R and intI2_F/intI2_R, for class 1 and 2 integrons respectively (Table 7.1). Isolates that gave a positive result using integrase specific primers were tested for the presence of inserted gene cassettes. Previously described primer sets 5'CS/3'CS and hep_F/hep_R (Table 7.1), with homology to the 5' and 3' conserved regions, were applied to detect and determine the size of the gene cassettes (for class 1 and class 2 integrons respectively). PCR products (5 µl) were analysed by electrophoresis on a 1 % agarose gel and stained with ethidium bromide.

7.2.6 Sequencing

PCR products (β-lactamase genetic determinants and integron gene cassettes) were purified with the JETQUICK PCR Purification Spin Kit (GENOMED, Löhne, Germany) and used as template in the sequencing reactions, carried out using an ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, California, USA). The ABI PRISM® 310 Genetic Analyser (PE Applied Biosystems) was employed for fragment separation and sequence analysis. The forward and reverse primers previously used for amplification of each DNA fragment were used in the sequencing reactions. On line similarity searches were performed with the BLAST software at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). β-lactamase genes and integron gene cassettes DNA sequences were translated using the Translate Tool available at the Swiss Institute of Bioinformatics' Expasy website (http://www.expasy.org/tools/dna.html). Sequence alignments were performed using the software Clustal X (Thompson *et al*., 1997).

Acession numbers: Nucleotide sequences determined for the fragments with homology (less than 97 %) with bla_{CphA} have been deposited in the GenBank database under the Accession Nos **DQ447638** and **DQ447639**.

Table 7.1 PCR primers used in this study

Primer pair	Target	Sequence (5'-3')	Annealing temperature (ºC)	Amplicon size (bp)	Reference
Carb_F	*bla*CARB	CAAGTACTTTYAAAACAATAGC	46	534	This study
Carb_R		GCTGTAATACTCCKAGCAC			
IntI1F	Class 1 integrase	CCTCCCGCACGATGATC	55	280	22
IntI1R		TCCACGCATCGTCAGGC			
IntI2F	Class 2 integrase	TTATTGCTGGGATTAGGC	50	233	21
IntI2R		ACGGCTACCCTCTGTTATC			
5'_CS	Class 1 integron variable region	AAGCAGACTTGACCTGA	58.5	variable	23
3'_CS		GGCATCCAAGCAGCAAG			
hep74	Class 2 integron variable region	CGGGATCCCGGACGGCATGCACGATTTGTA	60	variable	11
hep51		GATGCCATCGCAAGTACGAG			

7.3 Results

7.3.1 Prevalence of ampicillin resistant bacteria

Total CFU and ampicillin-resistant microorganisms were counted from water samples collected from three sampling sites in *Ria de Aveiro*, visited in the same day always at low tide. Ampicillin-resistant colonies accounted for 18 % (for site I-10), 14 % (for site I-6) and 15 % (for site N-1) of total CFUs (colony forming units) counted on MacConkey plates. In GSP plates the percentages of ampicillin-resistant CFUs were 72 % (for site I-10), 68 % (for site I-6) and 69 % (for site N-1) of the total CFUs.

7.3.2 Antibiotic resistance profiles

Sensitivity profiles were established by a disc diffusion method. Isolates were tested for susceptibility to a panel of 18 antibiotics. β-lactam antibiotics tested included penicillins (aminopenicillins, carboxypenicillins and ureidopenicillins), cephalosporins (1^{st}, 3^{rd}, and 4^{th} generations), monobactams and carbapenems. Additionally, another 4 classes of antibiotics were tested: tetracyclines, fluoroquinolones, aminoglycosides, and the combination sulphamethoxazole/trimethoprim. In Figure 7.1, the percentage of resistant strains to the panel of antibiotics tested is shown for the three sampling sites. In general the pattern of resistances found was similar for the three locations. Among ampicillin-resistant isolates, high resistance levels to other penicillins, in particular to carbenicillin and amoxicillin, were detected (Figure 7.1). Fifty-two out of 124 isolates (49 %) were resistant to at least two other antibiotics besides penicillins. Most frequently those additional resistances were to cephalotin (62 % resistant strains among *Enterobacteriaceae* isolates and 93 % among *Aeromonas* or *Pseudomonas* isolates) and tetracycline (48 % resistant strains among *Enterobacteriaceae* isolates and 12.5 % among *Aeromonas* or *Pseudomonas* isolates). Considerable levels of resistance to sulphamethoxazole/trimethoprim were also detected particularly among isolates identified as *Enterobacteriaceae* (23 %).

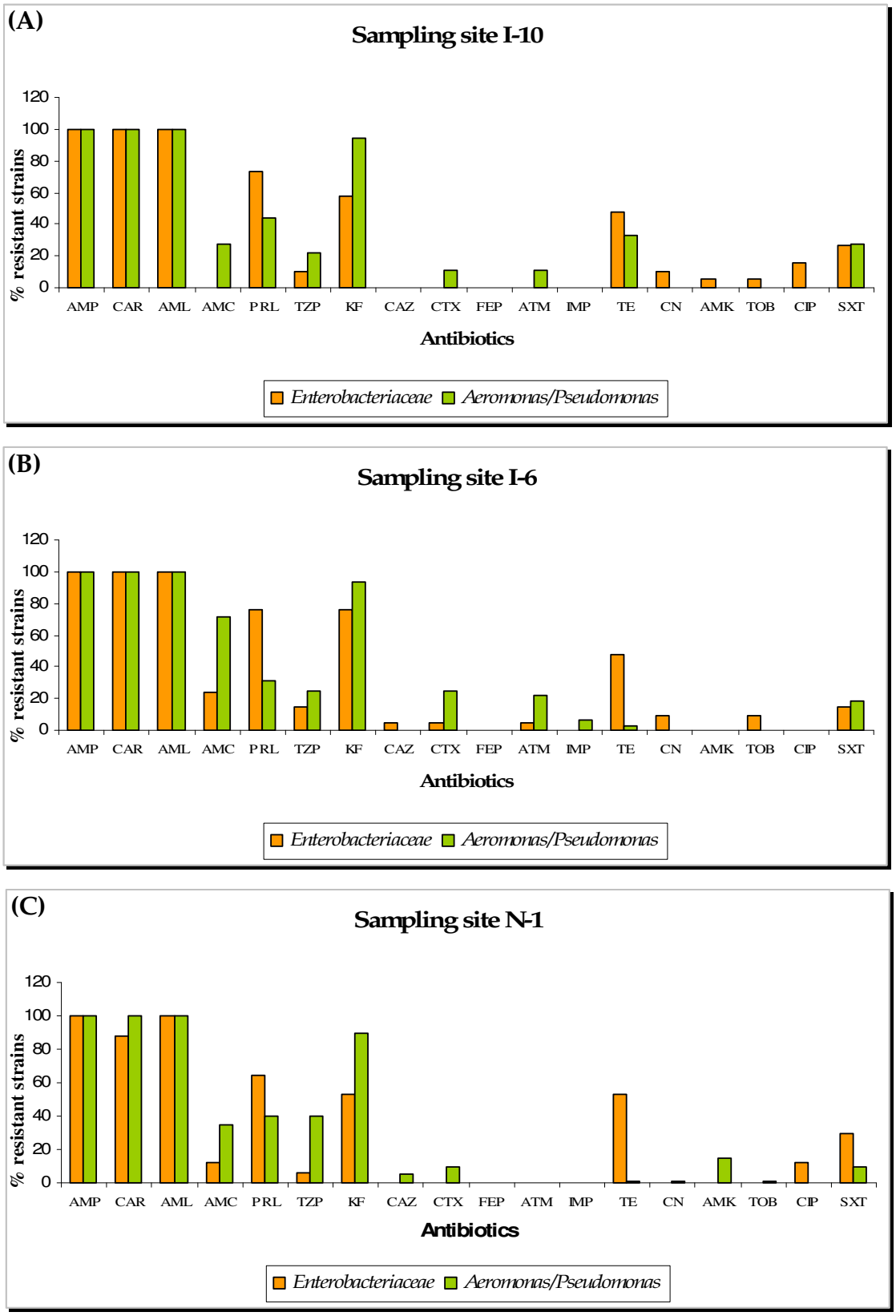

Figure 7.1 Antimicrobial resistance of ampicillin-resistant isolates from water samples collected from sites I-10 (A), I-6 (B) and N-1 (C).

7.3.4 Occurrence and diversity of β-lactamase genes

Occurrence of β-lactamase genes among ampicillin-resistant isolates was detected by PCR amplification, using primer sets targeting conserved regions of β–lactamase encoding sequences. Positive and negative controls were included in each experiment. Results obtained for isolates that gave a positive signal in at least one PCR experiment are summarised in Table 7.2. At least one β-lactamase gene was detected in 77.8 % of the *Enterobacteriaceae* isolates and in 10.5 % of the *Aeromonas* isolates.

The most frequently detected gene was bla_{TEM}, present in 36 isolates affiliated to genera *Escherichia* or *Shigella* (33 isolates), *Aeromonas* (2 isolates) and *Citrobacter* (1 isolate). Nucleotide sequence analysis revealed the presence of 4 sequence types displaying high percentage similarity (99 %) with the corresponding region of bla_{TEM-1}. Deduced amino acid sequence of the 4 nucleotide sequences displayed 100 % homology with TEM-1 (Boissinot *et al*., 1987).

Gene bla_{SHV} was detected in 8 isolates affiliated to genera *Klebsiella* (6 isolates), *Escherichia* (1 isolate) and *Aeromonas* (1 isolate). Sequence analysis revealed the presence of 5 nucleotide sequence types resulting in 3 different amino acid deduced sequences sharing 100 % homology with the following enzymes: OKP-A (for sequences retrieved from isolates M.I10.31, M.I10.42 and M.N1.26), OKP-B (for sequence retrieved from isolate M.I6.36) and SHV-1 (for sequences retrieved from isolates M.I10.33, M.I6.19, G.I6.24 and M.N1.23) (Fevre *et al*., 2005b; Livermore, 1995).

Finally gene bla_{CARB} was detected in 1 *Shigella* sp. isolate (identified as *Escherichia coli* using phenotypic methods), gene bla_{OXA-B} was detected in 2 *Aeromonas* sp. isolates and gene bla_{CphA} was detected in other 2 *Aeromonas* sp. isolates. Sequences encoding CARB and OXA-B genes were carried by class 1 integrons (see below). Sequence analysis of the entire cassettes revealed 100 % homology with genes bla_{CARB-2} (for isolate M.I10.10) and bla_{OXA-2} (for isolates G.I10.16 and G.N1.15) (Boissinot *et al*., 1987; Huovinen *et al*., 1988). Deduced amino-acid sequences from fragments amplified using primer set AER_F/AER_R from isolates G.I10.28 and M.I10.35 shared only 94 % homology with each other and 97 % with previously reported CphA β-lactamases (Massidda *et al*., 1991). These two nucleotide sequences were included in the GenBank database under accession numbers **DQ447638** (for isolate G.I10.28) and **DQ447639** (for isolate M.I10.35).

Only in one case two β-lactamase genes were detected in the same isolate (bla_{OXA-B} and bla_{TEM} in isolate G.I10.16). PCRs specific for CTX-M, IMP, VIM, OXA-A and OXA-C encoding sequences yielded no evidence for the presence of these genes in any isolate.

7.3.5 PCR detection of integrons and sequencing analysis of the corresponding variable regions

The presence of integrons was detected by PCR amplification of the *intI1* and *intI2* genes using previously described PCR primers (Table 7.1). Results are summarised in table 7.2. The *intI1* gene was present in 16 out of 54 *Enterobacteriaceae* isolates screened (29.6 %) and in 12 out of 57 *Aeromonas* isolates screened (21 %). The *intI2* gene was present in 4 isolates identified as *Escherichia/Shigella* (3 isolates) and *Citrobacter* sp. (1 isolate), isolated from sites I-10 (2 isolates) and I-6 (2 isolates). Isolate M.I10.40, identified as *Escherichia* sp., possess both *intI1* and *intI2* genes.

The 31 integrons, comprising *intI1* or *intI2* gene, were analysed for the presence of inserted DNA in the variable regions by PCR amplification using primer sets 5'_CS/3'_CS (for class 1 integrons) and hep_F/hep_R (for class 2 integrons) (Table 7.1). Seven integrons (all included in class 1) lacked any amplifiable gene cassettes in their variable region, presumably because of the absence of reverse primer target sequences. For the remaining integrons, partial direct sequencing of the amplified gene cassette regions was performed to confirm the presence of antibiotic resistance gene cassettes. Results obtained are summarised in Table 7.2. Thirteen different cassettes included in a total of 12 cassette arrays were identified. The majority of integrons (18) were shown to contain *aad*A-like gene cassettes (12 *aadA1*, 5 *aadA2* and 1 *aadA5*), which are known to confer resistance to streptomycin and spectinomycin. In 10 integrons an *aadA* relative was the only cassette contained in the variable region. Additionally, cassettes *dfrA1, dfrA7*, *dfrA12* and *dfrA17* (encoding resistance to trimethoprim), bla_{oxa-2} *and* bla_{CARB-2} (encoding resistance to β-lactams), *catB8* (encoding resistance to chloramphenicol), *sat1* (encoding resistance to streptothricin) and *orfD* and *orfF* (with unknown function) were detected. All 4 class 2 integrons carried the same cassette array (*dhfr1*, *sat1, aadA1*).

Table 7.2 Characterization of ampicillin-resistant strains isolated from three sites in *Ria de Aveiro* in what concerns resistance phenotypes, *bla* genotypes and integron content. Only the isolates that gave positive signal in at least one PCR experiment were included in this table.

Sampling Site	Isolate	Closest relative (% similarity)	Resistance phenotype[b]	β-lactamase gene content					Integrase	Putative gene cassettes[c]
				TEM	SHV	CARB	CphA	OXA-B		
I-10	M.I10.1	*Escherichia coli* (100 %)	AMP, CAR, AMX, PIP, TET	+	-	-	-	-	-	
	M.I10.2	*Escherichia coli* (99 %)	AMP, CAR, AMX, PIP, CEF, TET	+	-	-	-	-	-	
	M.I10.7	*Escherichia sp.* (99 %)	AMP, CAR, AMX, PIP, TET, GEN, CIP, SXT	+	-	-	-	-	I	*aadA1*[c]
	M.I10.8	*Shigella* sp./*Escherichia* sp. (99 %)[a]	AMP, CAR, AMX, PIP, TET, GEN,TOB, SXT	+	-	-	-	-	I	ND[d]
	M.I10.9	*Escherichia coli* (100 %)	AMP, CAR, AMX, PIP, CEF, TE, AMK	+	-	-	-	-	-	
	M.I10.10	*Shigella sonnei* (99 %)	AMP, CAR, AMX, PIP, TET, SXT	-	-	+	-	-	I	bla_{CARB-2}, *aadA2*
	M.I10.31	*Klebsiella pneumoniae* (100 %)	AMP, CAR, AMX	-	+	-	-	-	-	
	M.I10.32	*Shigella* sp./*Escherichia sp.* (99 %)	AMP, CAR, AMX, PIP, CEF, TET, CIP	+	-	-	-	-	I	ND
	M.I10.33	*Klebsiella pneumoniae* (100 %)	AMP, CAR, AMX, PIP, TZP, CEF	-	+	-	-	-	-	
	M.I10.34	*Shigella boydii*/*Escherichia coli* (99 %)	AMP, CAR, AMX, TET	+	-	-	-	-	I	ND
	M.I10.35	*Escherichia coli* (100 %)	AMP, CAR, AMX, PIP, CEF	+	-	-	-	-	-	
	M.I10.37	*Escherichia coli* (99 %)	AMP, CAR, AMX, PIP, CEF	+	-	-	-	-	-	
	M.I10.40	*Escherichia coli* (99 %)	AMP, CAR, AMX, PIP, TZP, CEF, TET, SXT	+	-	-	-	-	I, II	*dfrA1*, *aadA1* *dfrA1*, *sat1*, *aadA1*
	M.I10.42	*Klebsiella pneumoniae* (100 %)	AMP, CAR, AMX	-	+	-	-	-	-	
	M.I10.46	*Escherichia coli* (99 %)	AMP, CAR, AMX, PIP, CEF, TET, CIP, SXT	+	-	-	-	-	I	*dfrA17*, *aadA5*
	G.I10.6	*Escherichia coli* (99 %)	AMP, CAR, AMX, PIP, CEF	+	-	-	-	-	II	*dfrA1*, *sat1*, *aadA1*
	G.I10.8	*Aeromonas caviae* (99 %)	AMP, CAR, AMX, PIP, CEF, TET, SXT	-	-	-	-	-	I	*aadA2*
	G.I10.16	*Aeromonas sp.* (100 %)	AMP, CAR, AMX, CEF	+	-	-	-	+	I	bla_{OXA-2}, *orfD*
	G.I10.22	*Aeromonas caviae* (99 %)	AMP, CAR, AMX, PIP, TZP, CEF, TET, SXT	-	-	-	-	-	I	ND
	G.I10.25	*Escherichia coli* (99 %)	AMP, CAR, AMX, PIP, CEF	+	-	-	-	-	-	
	G.I10.27	*Aeromonas sp.* (99 %)	AMP, CAR, AMX, PIP, CEF	+	-	-	-	-	-	
	G.I10.28	*Aeromonas hydrophila* (100 %)	AMP, CAR, AMX, CEF, TET, SXT	-	-	-	+	-	I	*dfrA12*, *aadA2*
I-6	M.I6.1	*Escherichia coli* (99 %)	AMP, CAR, AMX, PIP, CEF, TET	+	-	-	-	-		
	M.I6.2	*Shigella* sp./*Escherichia* sp. (99 %)[a]	AMP, CAR, AMX, PIP, TET, GEN, TOB	+	-	-	-	-	I	*aadA1*
	M.I6.7	*Escherichia coli* (100 %)	AMP, CAR, AMX, PIP, CEF, TET, GEN, TOB	+	-	-	-	-	-	
	M.I6.10	*Escherichia sp.* (100 %)	AMP, CAR, AMX, PIP, CEF, TET	+	-	-	-	-	-	
	M.I6.11	*Escherichia sp.* (100 %)	AMP, CAR, AMX, PIP, CEF, TET	+	-	-	-	-	I	ND
	M.I6.19	*Escherichia coli* (99 %)	AMP, CAR, AMX, PIP, CEF, TET	-	+	-	-	-	I	*aadA1*
	M.I6.20	*Escherichia sp./Shigella sonnei* (99 %)	AMP, CAR, AMX, PIP, CEF, TET, SXT	+	-	-	-	-	II	*dfrA1*, *sat1*, *aadA1*
	M.I6.23	*Aeromonas* sp. (100 %)	AMP, CAR, AMX, AMC, PIP, CEF, SXT	-	-	-	-	-	I	*aadA1*
	M.I6.24	*Escherichia coli* (100 %)	AMP, CAR, AMX, PIP, CEF, TET	+	-	-	-	-	-	

	M.I6.26	*Aeromonas media* (100 %)	AMP, CAR, AMX, AMC, PIP, TZP,CEF, SXT	-	-	-	-	-	I	*dfrA7*
	M.I6.31	*Aeromonas* sp. (100 %)	AMP, CAR, AMX, AMC, CEF, TET	-	-	-	-	-	I	*aadA2*
	M.I6.32	*Citrobacter freundii* (100 %)	AMP, CAR, AMX, AMC, PIP, CEF, SXT	+	-	-	-	-	II	*dfrA1*, *sat1*, *aadA1*
	M.I6.33	*Shigella* sp. (99 %)	AMP, CAR, AMX, PIP, CEF	+	-	-	-	-	-	
	M.I6.34	*Shigella* sp./ *Escherichia sp.* (99 %)	AMP, CAR, AMX, PIP, TZP	+	-	-	-	-	-	
	M.I6.35	*Aeromonas hydrophila* (100 %)	AMP, CAR, AMX, AMC, CEF, IPM	-	-	-	+	-	-	
	M.I6.36	*Klebsiella pneumoniae* (100 %)	AMP, CAR, AMX	-	+	-	-	-	-	
	M.I6.37	*Shigella* sp./*Escherichia sp.* (99 %)	AMP, CAR, AMX, TET	+	-	-	-	-	-	
	M.I6.40	*Shigella* sp. (100 %)	AMP, CAR, AMX, AMC, PIP,CEF, TET, SXT	+	-	-	-	-	-	
	G.I6.14	*Aeromonas hydrophila* (99 %)	AMP, CAR, AMX, CEF	-	-	-	-	-	I	*catB8*, *aadA1*
	G.I6.24	*Aeromonas media* (99 %)	AMP, CAR, AMX, CEF	-	+	-	-	-	-	
	G.I6.30	*Aeromonas caviae* (100 %)	AMP, CAR, AMX, AMC, CEF	-	-	-	-	-	I	*aadA1*
N-1	M.N1.1	*Escherichia coli* (100 %)	AMP, CAR, AMX, PIP, CEF, TET, SXT	+	-	-	-	-	I	*dfrA12, orf F,aadA2*
	M.N1.4	*Escherichia coli* (99 %)	AMP, CAR, AMX, PIP, CEF, TET, SXT	+	-	-	-	-	I	*dfrA1*, *aadA1*
	M.N1.6	*Escherichia sp.* (100 %)	AMP, CAR, AMX, PIP, CEF, TET	+	-	-	-	-	I	ND
	M.N1.10	*Shigella* sp. (99 %)	AMP, CAR, AMX, PIP, CEF, TET, SXT	+	-	-	-	-	I	ND
	M.N1.16	*Shigella sp.* (100 %)	AMP, CAR, AMX, PIP, CEF, TET	+	-	-	-	-	-	
	M.N1.19	*Escherichia coli* (99 %)	AMP, CAR, AMX, PIP, TET, CIP, SXT	+	-	-	-	-	I	*aadA1*
	M.N1.23	*Klebsiella pneumoniae* (99 %)	AMP, CAR, AMX	-	+	-	-	-	-	
	M.N1.25	*Shigella sonnei* (99 %)	AMP, CAR, AMX, PIP, CEF, TET, CIP, SXT	+	-	-	-	-	I	*dfrA1*, *aadA1*
	M.N1.26	*Klebsiella pneumoniae* (99 %)	AMP, CAR, AMX, PIP, TZP	-	+	-	-	-	-	
	M.N1.28	*Escherichia coli* (100 %)	AMP, CAR, AMX, PIP, TET	+	-	-	-	-	-	
	G.N1.17	*Shigella* sp./*Escherichia* sp. (99 %)[a]	AMP, CAR, AMX, PIP, CEF	+	-	-	-	-	-	
	G.N1.15	*Aeromonas hydrophila* (99 %)	AMP, CAR, AMX, AMC, PIP, TZP,CEF, CTX	-	-	-	-	+	I	*aadA1*, *OXA-2*
	G.N1.20	*Aeromonas hydrophila* (100 %)	AMP, CAR, AMX, PIP, TZP, CEF	-	-	-	-	-	I	*aadA1*
	G.N1.27	*Aeromonas* sp. (100 %)	AMP, CAR, AMX, PIP, TZP, CEF	-	-	-	-	-	I	*aadA1*

[a] 16S rDNA sequencing did not allow to affiliate the isolate to only one genus

[b] Antibiotic abbreviations: AMP, ampicillin; CAR, carbenicillin; AMX, amoxicillin; AMC, amoxicillin-clavulanic acid; PIP, piperacillin; TZP, piperacillin-tazobactam; CEF, cephalothin; CTX, cefotaxime; CAZ, ceftazidime; IPM, imipenem; TET, tetracycline; GEN, Gentamicin; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole;

[c] Putative gene cassettes were identified by partial nucleotide sequencing and comparison with the sequences in the GenBank database by use of the BLAST service.

[d] ND, not detected.

7.4 Discussion

The role of natural environments as reservoirs of antibiotic-resistant bacteria has been addressed in several studies (Ash *et al*., 2002; Goñi-Urriza *et al*., 2000). In general those studies were designed to focus only the levels of bacterial resistance to a panel of antibiotics, and did not assess the presence and diversity of antibiotic resistance genetic determinants and mobile genetic elements. In general those studies were focussed on levels of bacterial resistance to a panel of antibiotics without assessing the presence and diversity of resistance genes and mobile genetic elements, two features that are important to understand the mechanisms of resistance and its spread. Studies regarding the environmental resistance gene pool are essential to clarify the importance of natural environments in the spread of antibiotic-resistance and to unravel the involved pathways.

Previous studies concerning the description of antibiotic resistance profiles of Gram-negative bacteria isolated from a variety of aquatic environments produced rather variable results. Even though, antibiotic resistance levels determined during this study were, on average, consistent with results reported by several authors (Ash *et al*., 2002; Goñi-Urriza *et al*., 2000; Roe *et al*., 2003; Rosser *et al*., 1999). Considerable differences in antibiotic resistance levels were registered between *Aeromonas, Pseudomonas* and *Enterobacteriaceae* isolates. This variability was expected since different intrinsic resistances were reported for these phylogenetic groups (Livermore, 1995). Also, as expected, high levels of resistance to other penicillins and cephalotin were detected among ampicillin-resistant isolates, probably due to the fact that resistance to penicillins and early cephalosporins is frequently conferred by the same resistance mechanism.[5] Resistance to tetracycline was also often registered, which is in accordance with previously published studies regarding Gram-negative isolates from aquatic environments and has been related to its extensive use (Goñi-Urriza *et al*., 2000). Tetracycline is frequently used in aquacultures to treat infectious diseases or in sub-therapeutic doses to prevent diseases (Chee-Sanford *et al*., 2001; Goñi-Urriza *et al*., 2000).

The presence of β-lactamase encoding sequences was detected in 77.8 % of the *Enterobacteriaceae* isolates and in 12.3 % of the *Aeromonas* isolates. *Enterobacteriaceae* isolates that gave no positive result in PCR experiments were identified as *Klebsiella oxytoca*, *Raoultella* sp., *Enterobacter* sp. and *Citrobacter freundii*. Among these, *K. oxytoca* and

Raoultella sp. strains are known to express class A β-lactamases that were not considered in this study (Fevre *et al*., 2005a; Walckenaer *et al*., 2004). In *Enterobacter* sp. and *Citrobacter freundii* resistance to β-lactams (namely penicillins and narrow spectrum cephalosporins) is generally attributed to the expression of chromosomal AmpC β-lactamases (Livermore, 1995). Also *Aeromonas* and *Pseudomonas* strains are known to possess a chromosomal bla_{AmpC} (Livermore, 1995) that may be responsible for the β-lactam resistance phenotype registered among isolates affiliated to these genera.

The most frequently detected gene was bla_{TEM}, followed by bla_{SHV}, $bla_{\text{OXA-B}}$, bla_{CphA} and bla_{Carb}. Deduced amino-acid sequences of the amplified fragments generally shared 100 % similarity with the corresponding regions of β-lactamases previously characterised from clinical isolates, namely with TEM-1 (a plasmid-encoded β-lactamase, previously characterized from a variety of clinical *Enterobacteriaceae* and *Aeromonas* isolates) (Boissinot *et al*., 1987), SHV-1 (also normally plasmid-encoded but found to be chromosomally mediated in *K. pneumoniae* isolates (Livermore, 1995)) and OKP-A and OKP-B (two chromosomal encoded β-lactamases recently described from *K. pneumoniae* isolates (Fevre *et al*., 2005b)). However, we are aware that, within each family, the diversity picture presented here may be distorted by the fact that only a region of each *bla* gene was amplified and analysed.

Primer set AER_F/AER_R allowed amplification of bla_{CphA} sequences from only two *Aeromonas* isolates, one of which exhibited imipenem resistance phenotype. The amino-acid composition deduced from nucleotide sequence of amplified DNA displayed only 97 % homology to previously described enzymes (Massidda *et al*., 1995). The high molecular variability of this gene (and probably also of primer target regions) may have resulted in false PCR-negative results.

During this study, two β-lactamase genes detected were contained in class 1 integrons: $bla_{\text{CARB-2}}$ (in isolate M.I10.10 identified as *Shigella sonnei*) and $bla_{\text{OXA-2}}$ (in isolates G.I10.16 and G.N1.15 both identified as *Aeromonas* sp.) (Table 7.2). These genes had been previously detected in class 1 integrons from *Enterobacteriaceae* or *P. aeruginosa* isolates (White *et al*., 2001). To our knowledge, this is the first report of integrons carrying OXA-type encoding sequences in *Aeromonas* isolates.

The *intI1* gene was present in 29.6 % of the *Enterobacteriaceae* isolates and in 21 % of the *Aeromonas* isolates. Rosser and co-workers reported that the incidence of class 1

integrons among Gram-negative bacteria isolated from estuarine waters was only 3.6 % (Rosser *et al*., 1999). Although the incidence levels obtained during this study are clearly higher, we should be cautious when comparing these results since bacteria here analysed were isolated for their ability to grow in the presence of ampicillin and there is a strong association between multiresistance and the presence of integrons (Roe *et al*., 2003; White *et al*, 2001). Even though, our results are considerably high also when compared with values obtained from resistant-selected strains from other aquatic environments (Roe *et al*., 2003).

Gene cassettes conferring resistance to several major classes of antibiotics were identified. The resistance conferred by the detected genes appears to be mainly towards "older" antibiotics such as early aminoglycosides and trimethoprim. As reported by other studies, the most frequently found resistance gene cassette was *aadA1*, being often the only cassette present (Roe te al., 2003; White *et al*, 2001). Also, gene cassettes encoding resistance to trimethoprim were frequently detected. This was also previously reported and discussed by other authors (White *et al*., 2001).

In conclusion, considerable levels of prevalence and diversity of β-lactamase genes were detected among ampicillin-resistant isolates collected from *Ria de Aveiro*. Also this study revealed that integron structures were prevalent in these bacteria, with the majority of the cassettes conferring resistance to antibiotics. These results support the hypothesis that estuarine environments constitute antibiotic resistance reservoirs. We should, however, emphasize that both prevalence and molecular diversity of the resistance genes under study were considerably lower than those found in clinical environments (French, 2005; Kotra & Mobashery, 1998; White *et al*., 2001). Most of the times, the genetic sequences here found represented ancient molecular variants, conferring resistance to older antibiotics. Evidently this probably results from different antibiotic selective pressures and we can conjecture that the increase in pollution levels in natural environments will enlarge and improve this resistance gene pool.

The present results concern exclusively culturable microorganisms selected in ampicillin-supplemented media. A few studies have already reported that cultivation-dependent approaches may underestimate the prevalence and diversity of antibiotic resistance genetic determinants in environmental samples (**Chapter 5**; Chee-Sanford *et al*., 2001; Schwartz *et al*., 2003). Our results support this hypothesis: based on cultivation-independent approaches we have found evidence for elevated levels of prevalence and

molecular diversity among β-lactamase encoding sequences in *Ria de Aveiro* (**Chapter 5**). With the present work, complementary information was obtained, namely about the phylogenetic groups carrying antibiotic resistance genes in this environment and also about the resistance phenotypes conferred by those genes.

8. *General discussion*

8.1 Phylogenetic diversity of bacterioplankton communities in Ria de Aveiro

The bacterioplankton communities within *Ria de Aveiro* have been extensively characterised in what concerns their abundance, several aspects of their metabolic activity and also the environmental factors responsible for controlling these parameters (see for example Almeida *et al*., 2002a; Almeida *et al*., 2002b; Cunha *et al*., 2000; Cunha *et al*., 2001). These studies concluded that communities reacted intensely and within short time intervals to shifts in environmental factors (Cunha *et al*., 2001). The environmental regulation of the activity was found to be mainly exerted by dissolved factors such as salinity and substrate availability (Almeida *et al*., 2002a; Cunha *et al*., 2000). The highest bacterial abundance and metabolic activity was registered in the middle section of the estuary with salinities ranging from 20 to 30 (Almeida *et al*., 2002a; Cunha *et al*., 2000). Moreover, the registered increase in activity rates in this section of the estuary (namely ectoenzymatic activity, productivity and uptake of monomers) was higher than the increase in cell abundance (Almeida *et al* 2002a; Almeida *et al*., 2002b; Cunha te al., 2001).

Taken together, results suggested the existence of phylogenetic diverse communities throughout the environmental gradients established in the estuary or alternatively the existence of communities with high metabolic adaptability to extremely dynamic environmental conditions. Phylogenetic information on these communities is needed to clarify the raised questions. Additionally, detailed insights into the phylogenetic diversity are essential to understand the shifts in the composition of prokaryotic populations and the links between composition and metabolic activity.

To our knowledge this study presents the first attempt to characterise the phylogenetic diversity of bacterioplankton communities within *Ria de Aveiro*. Two different culture-independent methodologies were applied: 16S rDNA libraries construction and analysis (**Chapter 3**) and DGGE (denaturing gradient gel electrophoresis) (**Chapter 4**). DGGE analysis revealed particularly helpful for the simultaneous analysis of a large number of samples allowing the clear identification of compositional shifts in the communities' structure. Analysis of 16S rDNA libraries displays a detailed picture of the phylogenetic composition of the communities but requires the scrutiny of a great number of clones from each sample. Moreover, analysis by DGGE involves only a small region (~200 bp) of 16S rRNA gene while clone library analysis involves a larger portion of the 16S rRNA gene (~550 bp) being more informative

in terms of the phylogenetic affiliations of the retrieved sequences. However, the clone library method is considerably more cumbersome and time consuming.

Results obtained by both methods were generally in agreement when comparing data acquired from the same sampling sites. However, some discrepancies were registered, namely the fact that clones affiliated with the *Bacteroidetes* group were only rarely retrieved from the 16S rDNA libraries whether clearly dominant in the DGGE analysis. As discussed before (**Chapter III**) this incongruence is probably due to the fact that, as described by other authors, the primer pair applied for the construction of the libraries underestimate this phylogenetic group (Glöckner *et al*., 1999). Furthermore the number of bands on the DGGE gels (18 bands on average for the I-6 samples and 20 bands on average for the N-1 samples) did not reflect the number of different sequences in the 16S rDNA clone library (25 OTUs for the I-6 site and 31 OTUs for the N-1 site) probably due to the co-migration in DGGE gels of a few non-identical fragments as has been reported before (Sekiguchi *et al*., 2001).

Both methods were previously suggested and applied to the study of bacterial communities in several different natural environments including estuaries (see for example Bouvier & del Giorgio, 2002; Castle & Kirchman, 2004; Covert & Moran, 2001; Crump *et al*., 2004). In this study phylogenetic diversity within the estuary was found to display similar aspects previously reported for other estuarine systems (Kelly & Chistoserdov, 2001; Rappé *et al*., 2000; Sekigushi *et al*., 2002), mainly in what concerns the dominant groups. Namely the dominance of *α-Proteoacteria* and *Bacteroidetes* groups in brackish and marine environments appear to be a common feature. Also the dominance of *β-Proteobacteria* and *Bacteroidetes* in the freshwater estuarine sections has been frequently reported.

Clear changes were identified in the phylogenetic composition of bacterial communities throughout the environmental gradient established in the estuary. Those changes were gradual and occurred essentially at lower phylogenetic levels within the marine and brackish sections of the estuary and were more drastic at the interface between the brackish and the freshwater sections of the estuary, where replacement of phyla occurred (**Chapter 3**; **Chapter 4**). Seasonal driven variability was also registered. Similar features have been reported for other estuaries (Bernhard *et al*., 2005; Crump *et al*., 2004; Hewson & Fuhrman, 2004). Also as reported for the same estuarine systems, salinity

was confirmed to be a key factor in determining microbial community composition shifts in *Ria de Aveiro* (**Chapter 4**).

Taken together, results suggest that phylogenetic differences are clearly relevant and greatly contribute to the different microbial activities registered in different sections of the estuary, but probably metabolic plasticity of the resident communities play also a key role, particularly in the marine and brackish sections. The phylogenetic data collected during this study besides being relevant to understand the functioning of the system, may be an important basis to design future projects concerning the monitorization of water quality in *Ria de Aveiro*, since bacterioplankton communities are highly responsive to environmental and anthropogenic pressures.

Specific biases associated with the applied methodologies were discussed in detail in the Chapters 3 and 4. Additionally, general limitations affecting culture-independent methodologies have been recognised. First of all, during an environmental study care must be taken to follow similar sampling procedures. Furthermore, we must be aware that DNA extraction efficiencies depend on lyses efficiency, which varies between microbial groups, and can also be affected by environmental characteristics as is the case of the presence of varied contaminants. In the same way, differential amplification of target genes can also bias PCR-based studies due to different affinities of primers to templates, different copy numbers of target genes, hybridisation efficiency and primer specificity (Farrelly *et al*., 1995; Kopczynski *et al*., 1994; Suzuki & Giovanonni, 1996).

In the study presented here care was taken to minimise the influence of the referred bias. Specifically the sampling procedure applied was always the same, additional lyses steps were included in the DNA extraction procedures and primers were carefully chosen. Even so, as discussed before some inconsistencies were detected when comparing results obtained using the two applied methodologies.

In conclusion we believe that the simultaneous use of different culture-independent methodologies with different degrees of resolution is the best way to obtain a broadest and less biased picture of the microbial community. Bacterial community compositional structure and dynamics should not be evaluated by only one method, and cross-check of the results obtained by several methods is essential.

8.2 Ria de Aveiro is a reservoir of antibiotic resistance?

The occurrence and diversity of β-lactamase genes in water samples collected from *Ria de Aveiro* were diagnosed during this study (**Chapter 5**; **Chapter 7**). β-lactamases were chosen as a model of study due to the fact that β-lactam substances are extensively used in clinical practice as well as in veterinary and agriculture, and also because these enzymes are extremely diverse in terms of their primary structure and of their hydrolytic capabilities (Demain & Elander, 1999; Kotra & Mobashery, 1998; Livermore, 1995). Most of the previously described β-lactamases are encoded in mobile genetic elements, raising the potential of dissemination of these antibiotic-resistance determinants (Livermore *et al.*, 1995). Taking this fact into consideration, occurrence and diversity of integrons (frequently described mobile genetic elements) among isolates from *Ria de Aveiro* were also assessed during this study (**Chapter 7**).

The prevalence of antimicrobial-resistant strains and antimicrobial-resistance genetic determinants in the environment is gaining worldwide concern (Alonso *et al.*, 2001; Kümmerer, 2003). Studies designed to evaluate the antimicrobial-resistance patterns in aquatic environments are crucial for the surveillance, prevention and control of the risk that such genes and/or microorganisms might constitute to human health and to ecosystem destabilisation. Also the obtained data should be considered when developing management plans to make waters safe for human use.

Ria de Aveiro estuarine waters are extensively used for human activities. For this reason, contamination of these waters with antibiotics or antibiotic resistant bacteria potentially constitutes a risk for human health. In fact, during this study several families of clinical relevant enzymes were detected indicating that *Ria de Aveiro* estuarine waters constitute a reservoir of β-lactamase genes, displaying high levels of molecular diversity. Surprisingly, even sequences encoding β-lactamases that confer resistance to last resort antibiotics, such as carbapenems, were detected. Also the prevalence of integrons was elevated. The high levels of resistance and the prevalence and diversity of resistance genes in *Ria de Aveiro* waters probably result from the impact of several polluting sources contaminated with unused or un-metabolised therapeutic drugs and also with resistant microorganisms. Other aquatic environments receiving contaminants from several different sources have been reported to be important reservoirs of antibiotic-resistant

microorganisms and also genetic determinants conferring resistance to a wide variety of antibiotics (Aminov *et al*., 2001; Roe *et al*., 2003; Rosser *et al*., 1999; Schwartz *et al*., 2003).

8.2.1 Culture-dependent versus culture-independent methods

The study of the ecology of β-lactamase genes in *Ria de Aveiro* was conducted using both culture-independent and culture-dependent methodologies. The results here reported revealed that, as previously suggested, culture-dependent methodologies underestimate the occurrence and diversity of antibiotic-resistance genetic determinants in natural environments (Chee-Sanford *et al*., 2001; Schwartz *et al*., 2003). In this study a higher number of β-lactamase families were detected in *Ria de Aveiro* waters when using culture-independent approaches than those detected by isolation methods. Moreover, the molecular diversity within each family was also higher among sequences retrieved by the culture-independent methodology.

However, as discussed above, culture-independent methodologies are subjected to several biases that should be considered when analysing results. Also, such approaches targeted DNA fragments which may not always correspond to functional enzymes and consequently may have no impact on effective resistance. On the other hand, despite the well known limitations, culture-dependent approaches allowed us to assess the phylogenetic affiliations of selected microorganisms carrying β-lactamase genes and also to characterise the resistance patterns conferred by these genes.

Consequently, we trust that to assess the potential of an environment to constitute a reservoir of antibiotic-resistance determinants several different methods should be applied. Preferably information must be gathered using methods based on cultivation and also culture-independent molecular methods.

8.3 Summary of conclusions

We believe that the results presented in this thesis are of fundamental value for understanding the complexity of estuarine ecosystems and that this study constitutes a lead for further investigation. Additionally this study highlights the fact that, as happens with other research subjects in microbiology, a polyphasic approach seems to be more appropriate to get a fairly accurate picture when studying highly complex environmental microbial communities. Results obtained allowed to point out some major conclusions concerning both phylogenetic diversity and the prevalence of antibiotic-resistance:

Phylogenetic diversity:

1. The pattern of prokaryotic diversity of bacterioplankton communities from *Ria de Aveiro* was comparable to other coastal and estuarine environments previously studied.

2. Compositional shifts within these communities occurred essentially between the brackish and freshwater sections. Less pronounced changes were also detected along the salinity gradient in the marine and brackish sections

3. Seasonally driven changes in microbial community in this estuary also occur.

4. Marine and brackish communities were clearly dominated by members of the *α–Proteobacteria, γ-Proteobacteria* and *Bacteroidetes* groups and freshwater communities were dominated by members of the *β–Proteobacteria, δ-Proteobacteria, ε-Proteobacteria* and *Bacteroidetes* groups.

5. Salinity and temperature accounted for a significant amount of the variability in the bacterioplankton community composition.

6. The combination of two different culture-independent molecular methods revealed useful to obtain a clearer and less biased picture of the community structure and dynamics.

Prevalence of antibiotic-resistance:

1. *Ria de Aveiro* was found to be a reservoir of molecular diverse putative β-lactamase encoding sequences.

2. Considerable levels of prevalence and diversity of β-lactamase genes and integrons were detected among ampicillin-resistant *Aeromonas* and *Enterobacteriaceae* isolates collected from *Ria de Aveiro*.

3. Most of the retrieved sequences were identical or very similar to gene sequences previously characterised from clinical isolates.

4. The patterns of molecular diversity found within the β-lactamase gene families studied using culture-independent methodologies do not correspond to those reported in studies focusing on clinical isolates.

5. Our investigations based on cultivation-independent approaches revealed higher prevalence and molecular diversity among β-lactamase encoding sequences in this estuary than revealed by culture-dependent methods. This fact reinforces the hypothesis that cultivation-dependent approaches underestimate the prevalence of antibiotic resistance genes in environmental samples and introduce some bias in the recovery of their molecular variants.

8.4 Future perspectives

Obviously, the study presented here raised a number of questions that remain open concerning the microbial communities in *Ria de Aveio*.

Despite the fact that important insights into the phylogenetic diversity of these communities were obtained, additional studies are needed to quantify the most prevalent phylogenetic groups. This information can be achieved using more quantitative analyses,

such as fluorescent in situ hybridisation (FISH), quantitative slot blot hybridisation or quantitative real time polymerase chain reaction (qRT-PCR) (Amann *et al*., 1995; Stahl *et al*., 1988; Zhang & Fang, 2006). Also links between phylogenetic and metabolic diversities must be established in order to attribute the measured microbial processes to a specific phylogenetic group. In recent years, a number of techniques have been suggested, which not only allow to identify specific metabolic activities of distinct phylogenetic entities within a community but also to ascertain environmental parameters that regulate the activity. Those techniques are for example combined microautoradiography and FISH (MAR-FISH or MICRO-FISH) and stable isotope probing of nucleic acids (SIP) (for a review see Gray and Head, 2001).

Further investigations are also needed to completely evaluate the potential of *Ria de Aveiro* waters to constitute a reservoir of potentially harmful antibiotic-resistant microorganisms or antibiotic-resistance genes. The search must be extended to genetic determinants conferring resistance to several groups of antibiotics, giving special relevance to the ones commonly used in agriculture, aquaculture and veterinary practices like tetracyclines. As stated before, we consider that culture-dependent and culture-independent methodologies must be combined to accomplish this task. Novel culturing procedures must be applied to assess a higher diversity of culturable bacteria and to determine not only their resistance phenotypes but also their genetic content in resistance determinants: in fact, resistance genes can be present but not expressed, thus constituting a reserve of new harmful phenotypes. Culture-independent methodologies based on RNA analysis would be useful to quantify the expression of antimicrobial resistance genes in the environment.

Finally we consider that the employment of metagenome-based technologies to the study of *Ria de Aveiro* bacterioplankton communities would be useful to characterise both phylogenetic and functional diversity (Handelsman, 2004; Riesenfeld *et al*., 2004b). The construction and analysis of environmental DNA libraries offers the possibility to isolate and study genes of interest such as resistance genetic determinants from uncultivable bacteria, and also to obtain phylogenetic information about the microorganisms carrying those genes.

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